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Genetic transformation of industrial species of Bacillus and Clostridium

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**Genetic transformation of industrial species of *Bacillus* and
*Clostridium***

Birrer, Gregory Andrew, Ph.D.

University of New Hampshire, 1991

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GENETIC TRANSFORMATION OF INDUSTRIAL SPECIES OF
BACILLUS AND CLOSTRIDIUM

BY

GREGORY A. BIRRER

BS Bethany College, 1984

DISSERTATION

**Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of**


Doctor of Philosophy

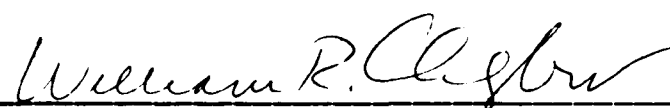
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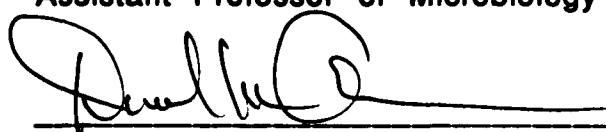
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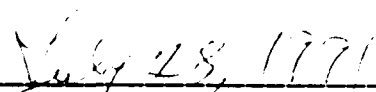

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DEDICATION

This dissertation is dedicated to my parents, Robert and Virginia Birrer, who taught me to believe that I can accomplish anything if I put my mind to it.

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I would like to thank my advisor Dr. Robert M. Zsigray for his continued support and advice as I differentiated from "green bean" status to a more fully conscious adult form. I also thank my co-advisor Dr. William Chesbro for his steady patience and support. His discussions, ideas and willingness to talk science anywhere (TP?) have already left their mark on me. I would also like to thank the other members of my committee; Dr. Donald M. Green, Dr. Andy Laudano, and Dr. Aaron Margolin. Thanks to Bob Mooney for expert technical and non-technical help, not to mention his friendship (just don't ask him for advice on where to go fishing). Robin, we made it! Thanks for the good times and getting me out of some bad ones. I would like to thank Linda Dibernardo and Alberta and Dick Moulton for helping me in more ways than they could imagine. They all made the department a much better place to live. Thanks also to all my fellow graduate students, past and present. Last but not least I want to thank Terri Sandelin Birrer. Without her love and help in all aspects of my life I may not have made it through this. I love you.

P.S. - Chief, be careful on Key Bridge late at night!

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ABSTRACT

GENETIC TRANSFORMATION OF INDUSTRIAL SPECIES OF *BACILLUS* AND *CLOSTRIDIUM*

by

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University of New Hampshire, September, 1991

The relationship between growth rate and competence for genetic transformation in *B. subtilis* 168 was investigated. *B. subtilis* grown in chemostat cultures expressed competence at a relatively rapid growth rate of 2.5 h (mass doubling time, t_d). Since the literature suggested competence normally occurred at slower growth rates in batch cultures, *B. subtilis* was grown in a biomass recycling fermentor and extremely slow growth rates were examined for the expression of competence under carbon/energy limitation. No additional peaks in the number of competent cells were detected. However, major metabolic manifestations were observed at the growth rate where competence is maximally

expressed. These were a change in steady state biomass concentrations precisely at the growth rate of maximal competence, and a change in the amount of acetate excreted as a metabolite of glucose underoxidation.

Data from the above studies were applied to studies in which a genetic transformation system was sought for *C. beijerinckii* NRRL B-592. No natural transformation was observed; consequently, a protoplast formation and regeneration system was successfully developed for this organism. This system, however, would not allow gene transfer by protoplast transformation or fusion.

Plasmid pHR106 DNA was introduced to intact cells of *C. beijerinckii* by electroporation and an analysis by Southern hybridization indicated that the plasmid had integrated into the chromosome. Recombinant derivatives of pHR106 harboring cloned inserts were tested for their ability to transfer and recombine into the chromosome after transfer to *C. beijerinckii*. These plasmids were transferred and expressed in *C. beijerinckii* cells, but may no longer integrate. The use of pHR106 and insert-containing derivatives to transform wild-type *C. beijerinckii* is discussed. The

plasmid-based transformation system outlined in these studies offers the best way yet described to introduce cloned genes to this organism.

INTRODUCTION

Microorganisms of the genus *Clostridium* share three characteristics; the formation of endospores, obligate anaerobic metabolism, and the inability to reduce sulfate to sulfite (Gottschalk et. al., 1981). This diverse genus can be broadly divided into two groups, proteolytic or saccharolytic, based upon substrate specificities. *Clostridium beijerinckii* is a saccharolytic bacterium with industrial potential because it can produce the commodity chemicals butanol and acetone or isopropanol as fermentation endproducts (Jones and Woods, 1987). This technique was originally studied and commercially implemented using *Clostridium acetobutylicum* to form the solvents acetone, butanol, and ethanol from sugar-containing feedstocks such as corn mash or molasses. This was the first successful large scale bacterial fermentation to be developed and is known as the acetone-butanol (AB) fermentation (Jones and Woods, 1986; Reed, 1988). The AB fermentation, essentially the same for either *C. acetobutylicum* or *C. beijerinckii*,

proceeds in two stages. In the acidogenic stage, acetic and butyric acids are the primary products of carbohydrate dissimilation. Secondary products that can also be found in the fermentation broth at this stage are lactic and propionic acids as well as ethanol (George et.al., 1983; Jones and Woods, 1986; McNeil and Kristiansen, 1986; Forsberg et. al., 1987; Ahmed et. al., 1988). As the fermentation progresses the second stage is reached wherein the culture switches to solvent formation. In the solventogenic stage, the primary endproducts are butanol, ethanol, and either acetone (*C. acetobutylicum* and some strains of *C. beijerinckii*) or isopropanol (*C. beijerinckii*) (George et. al., 1983; Ahmed et. al., 1988). Other secondary products such as n-propanol, or 1,2-propanediol, may be present in the fermentation broth at low concentrations depending on whether the substrate contains pentose (Forsberg et. al., 1987; Ahmed et. al., 1988). The precise stimulus for the switch from acids to solvents remains unclear and has been the subject of many investigations. Current hypotheses have implicated a number of environmental or physiological events as possible triggers of solventogenesis including; growth rate domain dependent metabolic changes at critical (slow) growth rates of the culture (Jones and

Woods, 1986; Terracciano and Kashket, 1986; Ahmed et. al., 1988; Ross et al., 1990), changes linked to an early phase of sporulation (Jones and Woods, 1986; Ross et al., 1990), and intra or extracellular pH (Terracciano and Kashket, 1986) . In any case, a significant difference exists between solventogenesis of *C. acetobutylicum* and that of *C. beijerinckii*. The latter organism is easily able to produce solvents at neutral pH, whereas the former has rarely been observed to do so (George and Chen, 1983; Ahmed et. al., 1988)

AB fermentation was the major method of producing acetone and butanol in Europe, Japan and North America from just after World War I until the 1950's (Jones and Woods, 1986; McNeil and Kristiansen, 1986). The fermentation became less competitive with and was eventually replaced by the chemical process of distillation from less expensive petro-chemical feedstocks (McNeil and Kristiansen, 1986). By 1965, the AB fermentation industry was nonexistent in Europe, North America, and Japan. Microbial production of these solvents continued in South Africa until 1983 and is still the major source of acetone and butanol in the Republic of China today where greater than 65,000 tons were produced in

1989 (Li et al., 1989).

There are a number of approaches to improving the AB fermentation by either *C. acetobutylicum* or *C. beijerinckii* that may render the process more economically competitive. Many attempts have been made to alter the environmental conditions affecting the physiology of the solventogenic organism (Jones and Woods, 1987). Much of what is now known concerning the conditions of solvent formation has been through such searches for optimum solvent yields. Studies have attempted to improve the solvent yield and reduce operating costs by discovering alternate, less expensive, starting substrates such as cheese whey, apple pomace, or Jerusalem artichoke mash (Jones and Woods, 1987). Another alternate feedstock would be waste cellulosic material. Cellulose is the most abundant naturally occurring source of polysaccharide on the planet and comprises about 30% of all agricultural waste (Lamed and Bayer, 1988). The ability to biologically degrade raw cellulose is rare in nature and is almost solely attributable to microbial action. At least three distinct enzymes are required to synergistically degrade crystalline cellulose, these are cellobiase, endoglucanase and exoglucanase enzymes (Lamed and Bayer, 1988).

While no solventogenic clostridia are known to possess all three enzymes, *C. acetobutylicum* has been shown to degrade soluble cellulose and xylans (Rogers, 1986).

Another approach that has been successfully employed with other industrial microorganisms such as *Bacillus* ssp., and *Streptomyces* ssp. is the isolation and selection of superior organisms through genetic manipulations (Reed, 1988). Classical and recombinant genetic techniques represent promising ways to improve the strains used in the AB fermentation. However, unlike genetically well characterized bacteria such as *Escherichia coli* or *Bacillus subtilis*, there is a comparatively small genetic database available on the clostridia. The requirement of clostridia for an anaerobic environment has evidently been a factor. More importantly, there has been no simple, generally applicable transformation system available for the introduction of exogenous DNA to these anaerobes. Consequently, the use of most established recombinant DNA techniques are precluded for most species of Clostridia (Jones and Woods, 1987; Reed, 1988).

The following section describes what achievements have been made in increasing the size of the genetic database for clostridial

species with an emphasis on the solventogenic clostridia. In addition, original research is presented that examines the physiology of natural transformation as it exists in the industrial microorganism *B. subtilis* as a prelude to understanding, developing, and exploiting a transformation-based gene transfer system for the solventogen *C. beijerinckii*.

I. LITERATURE REVIEW

State of *Clostridium* Genetics

Mutagenesis. In the past decade reports on genetic aspects of the clostridia have been increasing in the literature. Initial studies focused on developing efficient methods of DNA mutagenesis in order to generate auxotrophic and antibiotic resistant strains to facilitate cloning and transformation experiments (Reed,1988). One of the first clostridia examined, *Clostridium thermocellum*, was found to be refractory to mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and was best mutated by U.V. irradiation (Gomez et al.,1981). Walker (1983), however, found almost the opposite situation in *C. thermocellum* where U.V. irradiation only ineffectively mutated this species and ethyl methanesulfonate (EMS) was found to be a satisfactory mutagen. Bowring and Morris (1985) tested the efficacy of three agents, U.V. irradiation, MNNG and EMS to produce rifampicin resistant mutants of *C. acetobutylicum*. Of these methods U.V. irradiation produced the fewest mutants although prolonged exposure reduced cell viability. Exposure to MNNG (50

µg/ml) was the most toxic to the cells but did not produce as many mutants as did exposure to EMS (2 % v/v), even though cell viability was relatively unaffected by its presence (Bowring and Morris, 1985). Cavedon et al. (1990) treated vegetative cells of the cellulolytic *Clostridium* strain C7 with 500 µg/ml MNNG for 20 min at 30°C and successfully produced cellulase (avicellase) mutants. Other methods or chemicals were not evaluated in this study (Cavedon et al., 1990). In some cases, saccharolytic clostridia were found to be refractory to chemical mutagenesis by MNNG (Jones and Woods, 1987; Reed, 1988). The most successful mutagen of *Clostridium acetobutylicum* in a study by Lemmel (1985) was EMS, which produced a mutation rate that was 200-300 times that of background. Overall, EMS appears to be the most effective chemical mutagen for saccharolytic clostridia. Another advantage of EMS is its ability to penetrate clostridial endospores. In this way very large populations can be exposed and easily screened. U.V. irradiation has also been used with moderate success to generate various mutants of *C.thermocellum* but was relatively ineffective against *C. acetobutylicum* (Jones and Woods, 1987; Reed, 1988).

Recently, transposon mutagenesis of *C. acetobutylicum* has

been reported by several groups. Bertram et al. (1989) were able to pass the conjugative transposons Tn916 and 925 from *Enterococcus faecalis* to *C. acetobutylicum* strain DSM792 (Bertram and Durre, 1989). The transposons were plasmid borne and were transferred by conjugation at an average frequency of 5×10^{-2} per donor. Southern hybridization indicated that the transposon integrated into the chromosome at either single or multiple sites. After selection of transconjugants on tetracycline, no plasmid DNA was detectable in the cytoplasm (Bertram and Durre, 1990). Interestingly, they also found that Tn917, which carried an erythromycin (MLS) resistance gene, was not transferred and that Em was not a suitable marker for transposon mutagenesis of their strain of *C. acetobutylicum*. Tn916 mutagenesis was then used to generate 16 different solvent formation mutants. Three types of mutants were characterized: type I showed ethanol, no acetone or butanol; type II produced low amounts of each solvent; type III produced high amounts of butanol and low acetone and ethanol. These solvent-formation mutants could no longer sporulate (Bertram et al., 1990).

Woolley et al. (1989), reported successful transfer of Tn1545 and Tn916 to *C. acetobutylicum* strain NCIB 8052 (ATCC 824). The

multiple antibiotic resistant transposon Tn1545 inserted into the chromosome at several sites but the isolation of any particular mutants was not reported (Woolley et al., 1989).

Clostridium Plasmids. Many initial genetic studies involved screening various species of clostridia for naturally occurring plasmids in the hope they could eventually be used for the construction of cloning and/or shuttle vectors. Most reports encountered difficulties in extracting and detecting the plasmid DNA due to high DNase activity associated with the cells and in lysis-resistant cells (Blascheck and Klacik, 1984; Luczak et al., 1985; Lee et al., 1987; Reed, 1988; Mahoney et al., 1986; Popoff and Truffaunt, 1985). Plasmids have been found in most strains of the medically important proteolytic clostridia including, *C. perfringens*, *C. botulinum*, *C. tetani*, *C. difficile*, and *C. cochlearum* (Rogers, 1986; Lee et al., 1987). Functions have been ascribed to some of these plasmids. For example, plasmids from *C. cochlearum* provided resistance to heavy metals (Rogers, 1986). Plasmids from *C. difficile*, *C. tetani*, and *C. botulinum* contain various toxin genes (Lee et al., 1987). Strains of *C. perfringens* are known to harbor plasmids which code for a bacteriocin (Li et al., 1980), a gene responsible for

caseinase activity (Blascheck and Solberg, 1981), and several antibiotic resistance genes (Rood et al., 1978), including, tetracycline, erythromycin (macrolide, lincosamide, and streptogramin B antibiotics), and chloramphenicol, (Abraham et al., 1988; Berryman and Rood, 1989; Abraham and Rood, 1987). Plasmids have also been found in saccharolytic species such as *C. acetobutylicum*, *C. beijerinckii*, *C. butyricum* and *C. saccharoperbutylacetonicum*. In one survey, 7 of 21 solventogenic strains were found to contain plasmids, all of which were cryptic (Truffaut and Sebald, 1983). These investigators also reported the existence of a 2.6 megadalton cryptic plasmid in *C. beijerinckii* NRRL B-593. More recently a more extensive plasmid screening of *Clostridium* strains was carried out by Lee et al. (1987). They found one or more plasmids in 5 of 7 *C. acetobutylicum* strains tested. No plasmids were found in any of three strains of *C. beijerinckii* (DSM 53, 791, and 1820). Minton and Morris (1981) have described a strain of *C. butyricum* that harbors three cryptic plasmids, one of which was developed into a cloning vector.

Clostridial Shuttle and Cloning Vectors. Once clostridial plasmids were identified and characterized attempts

were made to use them to construct cloning and shuttle vectors. Initially, the lack of any reliable transformation system required clostridial vectors to be expressed in other bacterial systems such as *E.coli* and *B. subtilis*. Cloned clostridial genes were frequently characterized in these organisms. Since most clostridial 'cloning' vectors necessarily had to be shuttle vectors, these types of plasmids are discussed together and are shown in Table 1.

The first shuttle plasmid or cloning vectors for use in clostridia were constructed from the cryptic plasmids of *C. perfringens* strain VPI 12502 and were used in this species. Three different, small <4 kilobase (kb) cryptic plasmids were recombined with *E. coli* plasmid pBR322. Tetracycline resistance determinants from the *C. perfringens* plasmids pCW3, and pJU124 were cloned in these recombinant plasmids. The resulting plasmids could bestow tetracycline resistance upon *E. coli* cells (Squires et al., 1984). Later, after developing an L-form transformation system, they found the plasmids would also function in *C. perfringens* (Heefner et al., 1984). Another *E. coli* - *C. perfringens* shuttle plasmid, pHR106, (Table 1) was generated by Roberts et al. (1988). They combined the cryptic *C. perfringens* strain ATCC 12502 plasmid pJU122, to

Table 1. Clostridial shuttle Plasmid Constructs

Plasmid	Relevant Markers	Transfer Method	<i>Clostridium</i> Host	Other Host(s)	Reference
pJU12	Ap ¹ ,Tc	PT ²	<i>C. perfringens</i>	<i>Escherichia coli</i>	Squires et al., 1984.
pJU13	Ap ¹ ,Tc	PT ²	<i>C. perfringens</i>	<i>E. coli</i>	Squires et al., 1984.
pJU16	Ap ¹ ,Tc	PT ²	<i>C. perfringens</i>	<i>E. coli</i>	Squires et al., 1984.
pHR106	Ap ¹ ,Cm	E ³	<i>C. perfringens</i>	<i>E. coli</i>	Roberts et al., 1988.
pMTL500E	Ap ¹ ,Em	E ³	<i>C. acetobutylicum</i> ATCC 8052	<i>E. coli</i> / <i>Bacillus subtilis</i>	Oultram et al., 1988.
pAK201	Ap ¹ ,Cm	E ³	<i>C. perfringens</i>	<i>E. coli</i>	Kim and Blascheck, 1989.
pKNT11	Ap ¹ ,Em	PT ²	<i>C. acetobutylicum</i> N1-4081	<i>E. coli</i> / <i>B. subtilis</i>	Truffaut et al., 1989.
pKNT14	Ap ¹ ,Em,Tc	PT ²	<i>C. acetobutylicum</i> N1-4081	<i>E. coli</i> / <i>B. subtilis</i>	Truffaut et al., 1989.
pTY10	Ap ¹ ,Cm	TP ⁴	<i>C. acetobutylicum</i>	<i>E. coli</i>	Yoshino et al., 1990.
pSB92A2	Ap ¹ ,Cm	E ³	<i>C. perfringens</i>	<i>E. coli</i>	Phillips-Jones, 1990.

1 - The ampicillin resistance gene in all the plasmids is that of the pUC, or pBR *E. coli* plasmids. 2 - Protoplast Transformation.

3 - Electroporation, 4 - Tris-PEG (polyethylene glycol) transformation method.

provide a clostridial origin of vegetative replication, with sequences from pUC18 to provide a multiple cloning site. The resultant plasmid was then combined with pJIR62 (Abraham et al., 1985) to provide a chloramphenicol resistance gene from *C. perfringens*. pHR106 replicated autonomously in both hosts, conferring ampicillin resistance on *E. coli* and chloramphenicol resistance on *C. perfringens* (Roberts et al., 1988). Kim and Blascheck (1989) constructed an *E. coli* - *C. perfringens* shuttle vector which is very similar to pHR106, sharing even the same chloramphenicol resistance gene (Table 1). This plasmid was shown to replicate in *C. perfringens* strain 3624A.

The first report of hybrid plasmid vectors for a solventogenic *clostridium* was by Luczak et al., (1985) in which they constructed *C. butyricum* plasmids that were expressed in *E. coli* and *B. subtilis*. Small cryptic *C. butyricum* plasmids were cloned into a pBR322 plasmid which already carried the chloramphenicol acetyl-transferase (CAT) gene from *B. subtilis* plasmid pC194 (Luczak et al. 1985). These plasmids lacked any regions bestowing a clostridial origin of replication and thus were unable to replicate in strains of clostridia (Table 1).

Oultram et al. (1988) produced a cloning/shuttle vector by combining the streptococcal conjugative plasmid pAMB1 with the *E. coli* plasmid pMTL20 (Table 1). Plasmid pAMB1 was already shown to replicate and express its erythromycin resistance gene in *C. acetobutylicum* (Chambers et al., 1988). The resultant plasmid, pMTL500E, was capable of replicating in either *E. coli*, *B. subtilis*, and other Gram positive bacteria such as *Staphylococcus aureus* (due to the promiscuity of pAMB1), and was shown to replicate in *C. acetobutylicum* strain ATCC 8052 after transfer by electroporation (Table 1) (Oultram et al., 1988).

Different shuttle plasmid vectors that were expressed in *C. acetobutylicum* (*saccharoperbutylacitonicum*) strain N1-4081, *E. coli*, or *B. subtilis* were constructed by Truffaut et al. (1989). It was found that *B. subtilis* plasmid pIM13, and pT127, an *S. aureus* plasmid, would confer Em and Tc resistance in *C. acetobutylicum* after protoplast transformation. Shuttle plasmids were made by cloning each of these plasmids in turn with pBR322. The resulting constructs were more versatile as shuttle plasmids than other *Clostridium* vectors made previously because they functioned in either *E. coli* or *B. subtilis*, in addition to *Clostridium* (Truffaut et

al., 1989). Strain N1-4081, however, was constructed by chemical mutagenesis and was selected as an autolysin mutant that would regenerate on their media after the cells were converted into protoplasts (Reysset et al., 1987). Thus, these plasmids may be limited to expression only in this strain.

Another *C. perfringens*/*E. coli* shuttle vector was generated by Phillips-Jones (1990) as shown in Table 1. This vector combined an *E. coli ori V* and β -lactamase gene from plasmid pHG165, a chloramphenicol resistance gene from Tn 4451 (Abraham and Rood, 1987), and an *ori V* from the cryptic *C. perfringens* plasmid pCP1. The plasmid, pSB92A2, also contains a multiple cloning site (Phillips-Jones, 1990).

Recently, Yoshino et al. (1990) developed a shuttle vector for use with *E. coli* and a *C. acetobutylicum* strain. They isolated a 3 Kb cryptic plasmid from a laboratory strain of *C. acetobutylicum* and combined it with an *E. coli* promoter probe plasmid, pKK232-8, which carried a promoterless CAT gene. The resulting plasmid, designated pTY10, conferred Cm resistance in *E. coli* and was transferred to *C. acetobutylicum* where it apparently underwent several deletion events. Plasmids ranging in size from 4.0-7.6 Kb

were extracted from *C. acetobutylicum* (Yoshino et al., 1990).

Most of the cloning and shuttle vectors produced from clostridial DNA have been made for use in *C. perfringens* strains. This may reflect the fact that the first clostridial transformation systems were worked out in this organism. It remains to be seen if these plasmids will be expressed in solventogenic strains and other clostridia.

Gene transfer systems for the Clostridia

Transformation. Natural transformation occurs in some microorganisms, such as *B. subtilis*, *Haemophilus* and *Streptococcus ssp.* when they become naturally competent to bind, internalize, and integrate foreign DNA (Stewart and Carlson, 1986). The process is termed natural because no overt treatments are done to the cells. There have been no reports of this type of transformation for any of the clostridia.

Protoplast Regeneration and Transformation An initial way to achieve genetic recombination between organisms or to introduce foreign DNA to Gram positive bacteria, such as *Streptomyces* (Bibb et al., 1978) or *B. subtilis* (Chang and Cohen, 1979), has been protoplast fusion or transformation (Allcock et al.,

1982; Jones and Woods, 1986; Reed, 1988; Heefner et al., 1984). The creation and exploitation of a protoplast manipulation system generally follows a failure to achieve genetic transfer by more conventional means.

There have been many attempts, listed in Table 2, to adapt these methods to the clostridia. Only protoplast methods devised for solventogens will be discussed below. Allcock et al., (1982) first showed that cells of *C. acetobutylicum* (strain P262) could be made into cell wall-deficient bacteria (protoplasts) by treatment with lysozyme, and when plated on an osmotically-reinforced medium, could regenerate intact cell walls. The optimal osmotic stabilizer for producing viable protoplasts and for promoting regeneration on a solid medium was sucrose (0.5 M). Regeneration on solid *Clostridium* basal medium (CBM) (O'Brien and Morris, 1971) with 5% wt/vol gelatin was best when 25mM of both $MgCl_2$ and $CaCl_2$ were present in the protoplast-formation broth (Allcock et al., 1982). This system was later used to transfect the bacteriophage CA1 into protoplasts of *C. acetobutylicum* P262J after incubation with phage DNA for 2 h at 37°C. No fusogenic, or protoplast transformation-promoting agents such as polyethylene glycol (PEG)

were used in this instance (Reid et al., 1983).

Minton and Morris (1983) described a method for production and regeneration of protoplasts of *C. pasteurianum*, another solventogen. As shown in Table 2 they used lactose (15% wt/vol) as osmotic stabilant and lysozyme to digest the cell wall. The regeneration medium (RM) was based on CBM with 2% agar, 2% bovine serum albumin (BSA), 0.1 M N-acetylglucosamine (NAG), and a catalase solution (400 U/ml final), which was added to 'minimize the inadvertant introduction of oxygen'. The NAG was added to inhibit residual lysozyme activity and to stimulate cell wall growth by providing peptidoglycan precursors. This medium supported regeneration frequencies ranging from 0.1 to 10% of the total number of protoplasts (Minton and Morris, 1983).

Lin and Blaschek (1984) reported the transformation of heat-treated protoplasts of *C. acetobutylicum* strain SA-1 with pUB110 plasmid DNA. Protoplast formation and regeneration were accomplished using the method of Allcock et al., (1982). It was reported that only after the protoplasts were heated at 55°C for 15 min, not 5 min more or less, could transformation of 1 in 6.6×10^4 protoplasts be achieved using pUB110 DNA. The narrow window of

heat treatment which allowed transformation to occur was explained as being long enough to inactivate extracellular endonucleases (DNases), or an intracellular restriction system, and still short enough to allow the protoplasts to retain their viability (Lin and Blascheck, 1984). To date these results have not been repeated by other groups (Reysset, et al., 1987; Reed, 1988).

Protoplast formation/regeneration conditions were reported by Yoshino et al., (1984) for *C. acetobutylicum* strain N1-4 (Table 2). Various osmotic stabilizers successfully maintained the integrity of the protoplasts after formation by lysozyme, but only 10 and 12% wt/vol. PEG 4000 supported subsequent regeneration when plated on a medium containing 3% agar. Regeneration occurred at frequencies ranging between 0.1 and 1.0% (Yoshino et al., 1984).

Protoplast formation and regeneration of *C. tertium* strain A1 was described by Knowlton et al. (1984). The protoplasts, generated by lysozyme treatment, and stabilized in sucrose, grew either as protoplast colonies or were regenerated in an agar overlay containing 1% bovine serum albumin (BSA). The overlay and solid regeneration media contained 1.5% agar and 5% gelatin. Gelatin was shown to stimulate protoplast colony growth at 5% wt/vol. Greater

Table 2. Protoplast Manipulation Systems

<i>Clostridium</i> strain	Method of cell wall removal	Osmotic Stabilizer	Regeneration Frequency (%)	Capacity for Transformation	Reference

<i>C. acetobutylicum</i> P262	lysozyme	0.5 M Sucrose	80	yes ¹	Allcock et al.,1982; Reid et al.,1983; Jones et al.,1985
<i>C. pasteurianum</i>	lysozyme	15 % Lactose	10	--	Minton and Morris, 1983
<i>C. perfringens</i> VPI 11268	penicillin G	0.4 M Sucrose	--	yes ²	Heefner et al.,1984
<i>C. perfringens</i> VPI 11268	autolysins	0.4 M Sucrose	? ³	yes	Heefner et al.,1984
<i>C. acetobutylicum</i> SA-1	lysozyme	0.5 M Sucrose	80	yes ⁴	Lin and Blascheck, 1984
<i>C. acetobutylicum</i> N1-4	lysozyme	12% PEG 4000	1.0	--	Yoshino et al., 1984
<i>C. tertium</i> ATCC 19405	lysozyme	0.6 M Sucrose	8.0-90	--	Knowlton et al., 1984
<i>C. perfringens</i> ATCC 3626B	lysozyme	0.5 M Sucrose	5.1 %	--	Stal and Blascheck, 1985
<i>C. acetobutylicum</i> N1-4080	lysozyme and penicillin G	0.6 M Sucrose	1.0 %	--	Reysset et al., 1987
<i>C. acetobutylicum</i> B643	lysozyme	0.3 M Sucrose	8.0-25 %	--	Reilly and Rogers, 1987

¹ Protoplast fusion by this method was also reported by the same group. ² Transformation occurred in broth culture and was unquantifiable. ³ No frequency was given. ⁴ work not reproducible (Reed, 1989).

amounts decreased the recovery of protoplast colonies but did not affect regeneration at any concentration (Knowlton et al., 1984).

Protoplast regeneration of *C. acetobutylicum* was accomplished by Reyssset et al. (1987) when they isolated an autolysin-deficient strain (N1-4080). The parent strain produced autoplasts, (cell-wall deficient cells that appear in stationary phase cultures ie., they are not induced), but these would not regenerate back to the walled state. Greater than 99% protoplasts were formed from whole cells of this strain by simultaneous lysozyme and penicillin G treatment and were stabilized by 0.6 M sucrose. Solid regeneration medium contained, other than the tryptone-yeast extract agar of Ogata and Hongo (1979), 0.3 M sucrose, 2.5% wt/vol agar, 1 mM CaCl_2 , 1 mM MgCl_2 , and 0.5% BSA. When strain N1-4080 (Wild-type) protoplasts were plated on this medium, regeneration was variable and never more than 0.01% (Reyssset et al., 1987). Observations suggested an inhibition of regeneration by residual osmotically resistant colonies, possibly by the diffusion of an autolysin. An autolysin mutant was selected from EMS-mutagenized cultures by isolating osmo-tolerant variants from conditions conducive to autolysis. The strain, designated N1-

4081, was shown to harbor 30% of the autolysin activity of the parent strain, and regenerated at a reproducibly higher frequency of 1%. Regeneration was improved tenfold further by the addition of a known autolysin inhibitor, sodium polyanethole sulfonate (SPS), to the medium (Reysset et al., 1987). In contrast to many other studies where certain regeneration medium constituents were deemed most critical to regeneration, here the importance of autolysin activity as the dominant factor in protoplast regeneration was stressed.

When Reilly and Rogers (1987) found that none of the published procedures for clostridial protoplast regeneration would allow regeneration of *C. acetobutylicum* strain B643, they devised new methods. For this strain, lysozyme treatment in a medium stabilized with 0.3 M sucrose was best for protoplast formation. Magnesium and calcium levels were found to be critical to subsequent protoplast regeneration when they were present during protoplast formation at high concentrations (50 mM). A solid regeneration medium was also described which interestingly contained no evident osmotic stabilizer. It was reported that sucrose, gelatin, PEG, and lactose, all frequently used RM ingredients, actually reduced the number of regenerant cells.

Contrary to other reports, NAG, and BSA had no positive effect, and agar inhibited regeneration when present at high concentrations (>1.5 %). The optimal medium, based on CBM (O'Brien and Morris, 1971), consisted of soft agar (0.8%) with $MgCl_2$ and $CaCl_2$ at concentrations of 25 mM (Reilly and Rogers, 1987). They did not report use of the system for fusion or transformation.

The procedures used for protoplast formation and stabilization were relatively constant regardless of the strain(s) used in the above studies, however, the methods and media for regeneration varied. Many of the aforementioned investigators reported a failure to apply published protoplast manipulation protocols to other strains of *Clostridium* (Reysset et al., 1987; Reilly and Rogers, 1987). The conditions for efficient protoplast regeneration therefore are regarded as being relatively species-specific (Reilly and Rogers, 1987). In addition, few of these reports have led to demonstrable transformation, although this may reflect the fact that most of the more popular Gram positive cloning vectors are not expressed in some clostridial species.

Protoplast Fusion. Protoplast fusion involves the joining of two genetically distinct strains, and like protoplast transformation,

is usually mediated by a membrane fusion-promoting agent such as PEG (Hopwood, 1981). Resulting recombinants are usually characterized after selection and regeneration. The only report of clostridial protoplast fusion was that of Jones et al. (1985). They showed that two doubly auxotrophic mutants of *C. acetobutylicum* strain P262 could be recombined by fusion. Fusion was demonstrated to occur at a frequency of 0.2 - 3.0 %. Segregating biparentals were also observed. These were regenerated colonies that, when subcloned, gave rise to both parental types, or parental and recombinant types, and apparently were diploid. Biparentals were first observed and studied after *B. subtilis* protoplasts were fused, and later they were observed to occur in *Streptomyces* sp. (Hopwood and Wright, 1978). In either case, only one of the chromosomes is expressed at any given time (Hotchkiss and Gabor, 1980; Hopwood, 1981). Additionally, it was reported that recombinants could be isolated after subcloning and segregation of biparentals. These were termed late recombinants (Jones et al., 1985). No further studies of these phenomena have been undertaken.

Tris-PEG Transformation. There are two reports (Soutschek-Bauer et al., 1985; Yoshino et al., 1990) of

transformation of *Clostridium* strains by the Tris-PEG method of Takahashi et al. (1983). This method was originally devised by the latter group for transformation of whole cells of *Bacillus brevis* and a slightly modified version was also used by Heierson et al. (1987) to successfully transform many subspecies of *Bacillus thuringiensis* with pBC16 and pC194 plasmid DNA. Treatment of *B. brevis* cells with 50 mM Tris-HCL was previously shown to remove the two outer protein layers of the organism (Yamada et al., 1981). This allowed the cells to become competent and transformation was stimulated by subsequent treatment with PEG (Takahashi et al., 1983). Briefly, the procedure used for transformation of *C. acetobutylicum* by Yoshino et al. (1990) was as follows: log-phase cells were treated with 50 mM Tris-HCL buffer (pH 8.5) for 1 h at 37°C. The cells were then centrifuged and suspended in 1 ml of growth medium (TYA broth) into which was added the plasmid DNA and 1.4 ml of 40 % PEG 6000, and the mixture was incubated for another h at 37°C. The cells were plated on selective plates after being suspended in 1 ml of TYA growth medium for 2 h (Takahashi et al., 1983; Yoshino et al., 1990). This allowed Yoshino et al. (1990) to transfer the shuttle plasmid pTY10, and deletion derivatives pTYD101 and pTYD104 to

intact cells of *C. acetobutylicum*. The transformation efficiency of *C. acetobutylicum* was between 10 and 1000/ μ g plasmid DNA (pTY104).

Clostridium thermohydrosulfuricum DSM 568 was also reported to be transformed with plasmid DNA using this method by Soutschek-Bauer et al. (1985). Cells treated with Tris-HCL (pH 8.3) and PEG 6000 could be transformed with pUB110 and pGS13 at an average frequency of 4×10^{-6} (Soutschek-Bauer et al., 1985).

Transformation by Electroporation. Transformation of bacteria by electroporation became widely used during the late 1980's. This procedure, first used to fuse eukaryotic cells, relies on high voltage, low amperage, square or exponential decay wave-form electric discharges to transiently 'porate' the membranes of bacterial cells suspended in a high resistance liquid buffer of low ionic strength. The discharge allows small molecules such as proteins, nucleotides, or folded DNA molecules to enter or leave the cells (Dower et al., 1988). This relatively recent technique has opened the way for the genetic transformation of many relatively uncharacterized species of bacteria such as *Clostridium*. In addition, electroporation has increased the efficiencies of plasmid

transformation in species such as *E. coli* and *B. subtilis* which were previously transformable by other means (Dower et al., 1988). Like natural transformation, electro-transformation has the advantage that DNA can be passed into intact or untreated cells and therefore no extra manipulations are needed to recover recombinants.

The first report of electroporation-induced transformation of *Clostridium* was by Oultram et al. (1988), in which whole cells of *C. acetobutylicum* strain ATCC 8052 were transformed with the *E. coli*/*C. acetobutylicum* shuttle vector pMTL500E. The commercially available electroporation unit from Bio-Rad called the Gene Pulser™ was used. The most consistent transformation of this strain occurred when a cell-DNA mixture was pulsed at 5.0 KV/cm, using cells harvested from the mid-exponential growth phase with no special treatments prior to transformation. The frequency of transfer was between 8.0×10^1 and 2.9×10^3 transformants/ μ g plasmid DNA. Under these conditions the number of cells that survived the pulse were between 0.7 and 23 %. Also reported was the cloning of a *C. pasteurianum* leucine gene into pMTL500E and successful transfer to a leucine mutant of *C. acetobutylicum* (Oultram et al., 1988).

Kim and Blascheck (1989) observed a slightly higher frequency when they transformed *C. perfringens* ATCC 3624A with the shuttle plasmids pAK201 and pHR106 (Roberts et al., 1988) by electroporation. The same Bio-Rad apparatus was used to deliver the pulse. However, the field strength (KV/cm) used is uncertain because they neglected to report what type of cuvette was used (the inter-electrode gap distance of the cuvette is needed to correctly repeat the conditions). They do, however, report that 1×10^6 transformants/ μ g of plasmid DNA was achieved from late-log phase, untreated cells, but they do not specify how this number was obtained, or the extent of cell lysis after the shock was applied (Kim and Blascheck, 1989). In a later paper it was shown that in addition to the above 2 plasmids; pIP401 pAMB1, pVA1, and pVA677 were also transferred to *C. perfringens* 3624A by their methods (Allen and Blascheck, 1990).

A description of the conditions for electro-transformation of *C. perfringens* strain 13 with the Bio-rad unit is given by Scott and Rood (1989). Cells harvested from the early-log phase of growth were optimally transformable with plasmid pHR106 (2.5×10^{-4} transformants/viable cell/ μ g DNA). Transformation was observed

only after the cells had been pretreated with lysostaphin (2 - 20 $\mu\text{g/ml}$) for 1 h at 37°C and then subjected to a pulse of 6.25 KV/cm. It is interesting that three other strains of *C. perfringens*; CW504, JIR81, and ATCC 3626B, were untransformable by the same methods (Scott and Rood, 1989).

Another study of electroporation involving *C. perfringens* strain p90.22 was carried out by Phillips-Jones (1990). Plasmid pSB92A2 was transferred at the highest frequency when late-log phase cells were concentrated in 15% glycerol, combined with DNA, and pulsed at 6.25 KV/cm. It was indicated that this voltage caused the highest amount of cell death ranging from 99.5 - 99.75%. A 3 h post-shock incubation time was reported to provide optimal recovery of transformants, about 4.4×10^3 transformants/ μg plasmid DNA (Phillips-Jones, 1990).

Conjugation. Conjugation was one of the first avenues used to introduce various plasmids into solventogenic and other clostridia. Table 3 lists studies concerning conjugation of clostridial strains. Only conjugation in solventogenic strains will be discussed in detail.

Oultram and Young (1985) described conjugative transfer of

Table 3. Conjugation systems using Clostridia

Donor	Recipient	Plasmid (Marker)	Transposon	Reference
<i>Clostridium difficile</i>	<i>C. difficile</i>	(Tc)	? ¹	Smith et al., 1981.
<i>C. difficile</i>	<i>C. difficile</i>	(TC, MLS)	? ¹	Wüst and Hardegger, 1983.
<i>Streptococcus lactis</i>	<i>Clostridium acetobutylicum</i>	pAM81 (MLS)	-	Oultram and Young, 1985.
<i>Bacillus subtilis</i>	<i>C. acetobutylicum</i>	pAM81 (MLS)	-	"
<i>C. acetobutylicum</i>	<i>S. lactis</i>	pAM81 (MLS)	-	"
<i>C. acetobutylicum</i>	<i>B. subtilis</i>	pAM81 (MLS)	-	"
<i>B. subtilis</i>	<i>C. acetobutylicum</i>	pAM81:pOD1 (MLS)	-	Oultram et al., 1987.
<i>Enterococcus faecalis</i>	<i>Clostridium tetani</i>	(Tc) ²	Tn916	Volk et al., 1988.
<i>C. tetani</i>	<i>E. faecalis</i>	(Tc) ²	Tn916	"
<i>C. tetani</i>	<i>C. tetani</i>	(Tc) ²	Tn916	"
<i>E. faecalis</i>	<i>C. acetobutylicum</i>	pAM211, (Tc)	Tn916	Bertram and Dörre, 1989.
<i>E. faecalis</i>	<i>C. acetobutylicum</i>	pCF10, (Tc)	Tn925	"

Table 3. Continued

<i>E. faecalis</i>	<i>C. acetobutylicum</i>	pINY1275 (Tc, MLS)	Tn925,Tn917	"
<i>Escherichia coli</i>	<i>C. acetobutylicum</i>	pAM211	Tn916	Bertram et al., 1990.
<i>E. faecalis</i>	<i>C. acetobutylicum</i>	(Tc,Em,Kn) ³	Tn1545	Wooley et al., 1989.
<i>B. subtilis</i>	<i>C. acetobutylicum</i>	(Tc,Em,Kn) ³	Tn1545	"
<i>C. difficile</i>	<i>B. subtilis</i>	(Tc)	?	Mullany et al., 1990.
<i>E. coli</i>	<i>C. acetobutylicum</i>	pCTC1, (Em)	-	Williams et al., 1990.
<i>E. coli</i>	<i>C. acetobutylicum</i>	pCTC3, (Cm)	-	"
<i>E. coli</i>	<i>C. acetobutylicum</i>	pCTC511, (Em)	-	"

¹ - The resistance determinant was unknown. ² - Tn916 was transferred from the chromosome of the donor. ³ - Tn1545 is a multiple antibiotic resistance transposon.

the streptococcal plasmid pAMB1 by a filter mating procedure from either *Streptococcus lactis*, or *B. subtilis* to *C. acetobutylicum* strain ATCC 8052. The plasmid, which confers resistance to macrolide, lincosamide, and streptogramin B antibiotics (MLS^r) was used because of its extremely high level of promiscuity as shown by the ability of pAMB1 to replicate in a wide variety of Gram positive bacterial genera, including *Streptococcus*, *Lactobacillus*, *Staphylococcus*, and *Bacillus* (Leblanc et al., 1978; Landman and Pepin, 1982; Young et al., 1989; Oultram and Young, 1985). Briefly, donor *S. lactis* cells were combined with those of *C. acetobutylicum*, impinged onto filter membranes, incubated anaerobically for 24 h, and the cells were plated on selective media. When *B. subtilis* was the donor (or recipient) the combination and impingement steps were carried out aerobically, and the filters were incubated anaerobically on a complex medium supplemented with 72 mM NaNO₃ which, they reported, supported growth of *B. subtilis* anaerobically by operating as the terminal electron acceptor (Oultram and Young, 1985). The transfer frequencies reported ranged between 4.1×10^{-5} and 1.4×10^{-3} for *S. lactis* to *C. acetobutylicum*, and from 7.0×10^{-7} and 3.0×10^{-5} for transfer in the opposite direction, and between 4.0×10^{-7} and

3.4×10^{-6} for *B. subtilis* to *C. acetobutylicum*, with 1.2×10^{-8} and 3.0×10^{-6} being the range of frequencies in the opposite direction (Oultram and Young, 1985). A later publication by the same group showed that a smaller cloning vector called pOD1 could be transferred from *B. subtilis* to *C. acetobutylicum* by cointegrate formation with pAMB1 in the former donor strain before conjugative transfer to *C. acetobutylicum* ATCC 8052 by similar methods (Oultram et al., 1987). A failure of this plasmid to mobilize other small, co-resident, non-conjugative *B. subtilis* cloning plasmids (pHV33, pBC16, pUB110, pTV1) was also reported, the prohibitively low frequency of transfer (when the already low frequency (10^{-7} - 10^{-8}) of pAMB1 transfer to *C. acetobutylicum* is coupled to the frequency of mobilization of 1×10^{-3} of other plasmids by pAMB1) was cited as the reason. The rate of transfer of the cointegrate was similar to those reported for pAMB1 alone (Oultram et al., 1987).

Conjugative transposons such as Tn916, Tn918, and Tn925 were first identified in streptococci (Franke and Clewell, 1981), and characterized by Clewell and Gawron-Burke (1986). In particular, Tn916 has proven to be possibly the most promiscuous of the resistance elements (Bertram et al., 1990). This transposon has now

been documented to transfer to and be expressed in many genera including, *Bacillus*, *Clostridium*, *Staphylococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Alcaligenes*, *Escherichia*, *Citrobacter* (Bertram et al., 1990), *Listeria*, (Kathariou et al., 1987), and *Thermus aquaticus* (Sen and Oriel, 1990).

Bertram and Dürre (1989) showed that Tn916 (Tcr), Tn925 (Tcr), Tn 925::Tn917 (Tcr and MLSr), carried on plasmids pAM211, pCF10, and pINY1275, respectively, could be transferred to *C. acetobutylicum* strain DSM792 from *S. faecalis*. After the cells were combined they were incubated for 4 h in liquid broth, concentrated, spotted onto nitro-cellulose filter membranes, and incubated another 24 h before plating on selective media. This procedure resulted in transfer frequencies ranging from 7.3×10^{-4} and 4.5×10^{-3} transconjugants per donor input. If either the liquid or the solid-state incubation steps were omitted from the mating procedure the frequencies fell 100-fold. When the transconjugants were examined for plasmid content none of the plasmids were detected. Southern transfer and hybridization showed, in all cases, the determinants had integrated in the *C. acetobutylicum* chromosome. The pattern of integration of these transposons was

found to be random (Bertram and Dürre, 1989). Further experiments showed that *C. acetobutylicum* could act as the donor in intergeneric matings when the Tcr plasmid(s) were transferred to *B. subtilis*, and *S. lactis* subspecies *diacetylactis*. The frequency of transfer in these cases were lower, on the order of 1×10^{-7} per recipient. It is interesting that when Tn 925::Tn917 was transferred to *C. acetobutylicum*, the transfer frequency was reduced by 5 orders of magnitude if both Tc and Em were present in the selective plates as compared to Tc only plates. It was concluded that Tn917 was not as useful in this strain as the Tcr-conferring Tn916 and Tn925 and the latter two would be useful for mutagenesis (Bertram and Dürre, 1989). This group later reported the isolation of various Tn916 insertional mutants of *C. acetobutylicum*, some of which were impaired in solvent-formation (Bertram et al., 1990). Bertram et al. (1991) further showed that Tn916 could be transferred from various Gram-positive bacteria to various Gram-negative bacteria and vice-versa, including from *E. coli* to *C. acetobutylicum*, which occurred at a frequency of 1.4×10^{-5} per donor.

Woolley et al. (1989), reported transfer of Tn916, as well as the multiple antibiotic resistance-conferring *Streptococcus*

pneumoniae transposon Tn1545, (Courvalin and Carlier, 1986), from *E. faecalis* or *B. subtilis* to *C. acetobutylicum* NCIB 8052.

Conjugation experiments were carried out by incubating cell mixtures on nitrocellulose filter membranes for 4 - 16 h anaerobically. After the apparent transfer of Tn916 to this strain of *C. acetobutylicum* the authors reported an inability to subculture transconjugant colonies growing on selective plates containing Tc. It was thought that perhaps the transposon insertionally inactivated a vital gene. The frequency of transfer of Tn1545 (Tcr, Emr, Knr) from either *B. subtilis* or *E. faecalis* varied between 1 and 4 X 10⁻⁵ per donor. Tn1545 was carried in and transferred from the chromosome of these strains (not from a plasmid). Interestingly, if both Em and Tc were used in the selective plates, as opposed to using one or the other antibiotic, the transfer frequency was reduced by 100-fold (Woolley et al., 1989). This result is very similar to that reported by Bertram and Durre (1989). Southern blotting and hybridization of transconjugant *C. acetobutylicum* chromosomal DNA revealed that Tn1545 integrated in a random fashion and in some cases was present in multiple copies (Woolley et al., 1989).

Finally, Williams et al. (1990), describe a conjugative plasmid

transfer system from *E. coli* to *C. acetobutylicum* NCIB 8052. They developed three conjugative cloning vectors using the replicons from pAMB1 (*E. faecalis*), pCB101 (*C. butyricum*), and pWV01 (*S. cremoris*), combined with the *cis*-acting *oriT* gene of the *E. coli* IncP plasmid RK2. Other necessary conjugation functions were provided *in trans* on another IncP plasmid in the *E. coli* donor. A filter mating system provided transfer frequencies ranging between 2.0×10^{-6} and 6.1×10^{-7} transconjugants per donor. The authors state this system may be a useful alternative to electroporation-induced transformation and may work with other species of *Clostridium* (Williams et al., 1990).

Transduction. Although bacteriophages have been described in different species of *Clostridium*, and industrial AB fermentation runs were frequently shut down due to prophage infection, there have been no reports of transduction (Young et al., 1989). Two bacteriophages of *C. acetobutylicum*, CA1 and HM3 have been successfully used as a source of 'homologous' DNA for transfection experiments (Reid et al., 1983, Reyssett et al., 1987).

II. MATERIALS and METHODS

Bacterial strains and Growth Conditions

All strains used in this study and their sources are listed in Table 4. *B. subtilis* strains were maintained as spore stocks in 0.85% saline at 5°C, and *C. beijerinckii* strains were maintained as spore stocks in sterile 1:1:1 mixtures of potting soil, sand and CaCO₃. *B. subtilis* cultures were started by heat-shocking spore samples in LB broth at 100°C for 10 min and were incubated at 35°C overnight with shaking. Suspensions of *C. beijerinckii* spores in sterile modified George and Chen broth (GC) (George and Chen, 1983) were heat-shocked for 10 min at 80°C and grown overnight in either GC, or Clostridial Basal Medium, (CBM), (O'Brien and Morris, 1971) broth under stringent anaerobic conditions. *C. acetobutylicum* and *C. perfringens* strains were stored frozen at -70°C in 10% glycerol. Most manipulations involving clostridial cells or protoplasts were carried out in a Coy anaerobic hood (Coy Laboratory Products Inc., Ann Arbor, MI) at a mean temperature of 32 ± 3°C. Certain manipulations such as centrifugation or electroporation required the

Table 4. Bacterial Strains

Strains	Genotype	plasmid(s)	Source
<i>Bacillus subtilis</i>			
168	wild-type	none	OE Landman
168 <i>trpC2</i>	<i>trpC2</i>	none	OE Landman
<i>Clostridium acetobutylicum</i>			
ATCC 10132	(-)	none	ATCC
ATCC 4259	(-)	none	ATCC
<i>Clostridium beijerinckii</i>			
B-592	acetone	none	Nakamura
B-593	propanol	none	Nakamura
B-592C14	C ^{mr}	pHR106 [*]	this study
B-592E1	C ^{mr} , E ^{mr}	pRZE4 [*]	this study
B-592T1	C ^{mr} , T ^{cr}	pRZL3 [*]	this study
<i>Clostridium perfringens</i>			
VPI 12502	clinical isolate	3 cryptic	I. Roberts
<i>Escherichia coli</i>			
BD 792	F-, λ-, "clean strain"	cured	B. Bachman
DH5α	F-, Φ80d/ <i>lacZ</i> ΔM15 Δ(<i>argF</i> — <i>lacZYA</i>) U169, λ-, <i>endA1</i> , <i>hsdR17</i> (r- k,m+k), <i>supE44</i> , <i>thi1</i> , <i>recA1</i> , <i>gyrA96</i> , <i>relA1</i>	none	D. Hannahan

HB101	<i>hsdS20</i> , (r-b,m-b), <i>recA13</i> , <i>ara14</i> , <i>proA2</i> , <i>lacY1</i> , <i>galK2</i> , <i>rpsL20</i> , <i>xyf5</i> , <i>supE44</i> , <i>mtl</i> , <i>leu</i> , <i>mcrB</i> , F-, λ -.	none	B. Bachman
HB101-A	c.f. HB101	pHR106	this study
HB101-B	c.f. HB101	pJIR71	this study
HB101-C	c.f. HB101	pJIR229	this study
HBAEC-1 (HB101)	c.f. HB101	pRZE4	this study
HBL-3 (HB101)	c.f. HB101	pRZL3	this study
JIR1591 (DH5 α)	c.f. DH5 α	pJIR71	J.I. Rood
JIR810 (MM294)	<i>endA1</i> , <i>hsdR17</i> , (r-,m+) <i>recA1</i> , <i>relA1</i> , <i>supE44</i> , <i>thi1</i> , F-.	pJIR229	J.I. Rood

* Denotes part or all of the plasmid has integrated into the chromosome.

removal of cells from the anaerobic hood for short periods of time. All *E. coli* strains used in this study (Table 4), were maintained as frozen cultures in 8 % dimethyl sulfoxide (DMSO) or 10 % glycerol. Stocks were thawed and grown overnight in LB broth at 37°C with shaking.

Chromosomal DNA Preparations

Chromosomal DNA was extracted from *Bacillus* or *Clostridium* strains by the method of Marmur, (1961). DNA from bacteriophage 41C (Zsigray et al., 1973) was extracted by the method of Mandell and Hershey, (1960).

Plasmids and extraction procedures

Plasmids used in this study and their sources are listed in Table 5. The media and antibiotic levels used to maintain selective pressure on strains harboring plasmids used in this study are outlined in Table 6. Plasmids were extracted from *E. coli* and *B. subtilis* by the alkaline lysis method of Birnboim and Doly (1979). In addition, the rapid boiling method of Holmes and Quigley (1981) was used exclusively for plasmid extraction from *E. coli* because it required less time and resulted in cleaner preparations. A modification of this method was used to extract plasmid DNA from

Table 5. Plasmids

Plasmid	Marker	Transposon	Host	Replicative origin	Source
pTV1	Cm,Em	Tn917	<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	P. Youngman
pTV32ts	Cm,Em	Tn917	<i>B. subtilis</i>	<i>S. aureus</i>	P. Youngman
pTV53	Tc,Cm,Em	Tn917	<i>B. subtilis</i>	<i>S. aureus</i>	P. Youngman
pC194	Cm	—	<i>B. subtilis</i>	<i>S. aureus</i>	B.G.S.C. ¹
pE194	Em	—	<i>B. subtilis</i>	<i>S. aureus</i>	B.G.S.C. ¹
pUB110	Kn	—	<i>B. subtilis</i>	<i>S. aureus</i>	B.G.S.C. ¹
pBC16	Tc	—	<i>B. subtilis</i>	<i>Bacillus cereus</i>	C. Thorne
pIM13	MLS	—	<i>B. subtilis</i>	<i>B. subtilis</i>	I. Smith
pLS20	MLS	Tn917	<i>B. subtilis</i>	<i>Bacillus anthracis</i>	C. Thorne
pAM120	Tc	Tn916	<i>Escherichia coli</i>	<i>Enterococcus faecalis</i> ²	D. B. Clewell
pHR106	Cm/Ap	—	<i>E. coli</i>	<i>Clostridium perfringens</i>	P. B. Hylemon
pRZL3	Cm,Tc/Ap	—	<i>E. coli</i>	<i>Clostridium</i> / <i>E. coli</i>	this study
pRZE4	Cm,Em/Ap	—	<i>E. coli</i>	<i>Clostridium</i> / <i>E. coli</i>	this study

¹ - Bacillus Genetic Stock Center. ² - *E. faecalis* is the origin of Tn917 which resides on an *E. coli* plasmid.

Table 6. Plasmid Selection Conditions

Plasmid	Medium ¹	Antibiotic ²	Concentration (µg/ml)	Bacterial host
pHR106	LB	Ap, Cm	100, 10	<i>Escherichia coli</i>
"	GC	Cm	30	<i>C. beijerinckii</i>
pJIR229	LB	Ap, Em	100, 30	<i>E. coli</i>
pJIR71	LB	Ap, Tc	100, 10	<i>E. coli</i>
pRZL3	LB	Ap, Cm, Tc	100, 10, 10	<i>E. coli</i>
"	GC	Cm, Tc	30, 10	<i>C. beijerinckii</i>
pRZE4	LB	Ap, Cm, Em	100, 10, 30	<i>E. coli</i>
"	GC	Cm, Em	30, 15	<i>C. beijerinckii</i>

¹ - LB stands for Luria-Bertani medium (Maniatis et al., 1982), and GC for George and Chen medium (George et al., 1983). ² - Ap, Cm, and Em are abbreviations for ampicillin, chloramphenicol, and erythromycin, respectively

clostridial strains as follows: 100 ml early log-phase cultures of *C. beijerinckii*, or *C. perfringens* were centrifuged at 15,000 X g for 10 min in 45 ml Oakridge tubes and resuspended in 1/10 volume of TE buffer (10 mM Tris, 50 mM EDTA pH 8.0) containing 1 mg/ml lysozyme. After 30 min at 37°C the cells were transferred to 1.5 ml microcentrifuge tubes, centrifuged in an Beckman microfuge™ for 2 min at maximum speed, and the pellets were resuspended in 0.42 ml STET buffer (8.0 % glucose, 0.5 % Triton X-100, 50 mM EDTA, and 10 mM Tris pH 8.0). The tubes were then placed in boiling H₂O for 50 s and promptly centrifuged at maximum speed in a microcentrifuge at R.T. for 6 min. The supernate was then transferred to another tube and either 3 volumes of cold 95 % ethanol, or 1 vol of cold 3 mM Na acetate in isopropanol was added. The tubes were placed at -20°C for 1 h to precipitate the DNA. Precipitated DNA samples were centrifuged at maximum speed for 10 min in a microcentrifuge; the pellets were washed with 1 ml 70 % ethanol, dried under vacuum, and resuspended in TE buffer or H₂O for later use. Plasmid extractions from *Clostridium* strains were always carried out using a control strain of *C. perfringens* VPI 12502 (Table 4) which harbors three cryptic plasmids.

Transformation, transfection and electroporation

Transformation and transfection procedures used for *B. subtilis* were carried out as follows: cell samples (0.9 ml) were added to tubes containing 0.1 ml of a 100 ug/ml solution of chromosomal, phage, or plasmid DNA. The mixtures were incubated at 35°C with shaking for 15 min except when plasmid pTV32Ts DNA was used, in which case incubation was at 32°C (Youngman et al., 1985). Samples were diluted in Spizizen's salts (Anagnostopoulos & Spizizen, 1960) and prototrophic transformants were selected on Spizizen's minimal plates supplemented with 0.5% acid hydrolyzed casein (AHC), (United States Biochemical Corp.). The degree to which the cells were observed to transform to prototrophy after this series of manipulations was considered to be proportional to the fraction of competent cells in the original batch or continuous culture. Control plates were made by spreading 0.1 ml samples of non-competent cells and 0.1 ml DNA samples on selective plates.

Transfection of *B. subtilis* with bacteriophage DNA was assayed by adding 0.1ml of sample on TYS plates supplemented with 0.01M CaCl₂ (Zsigray et al., 1973). This was overlaid with 2.5 ml of TYS soft agar seeded with *B. subtilis* 168 wt to 10⁷ cfu/ml so the

plaques could be visualized. The plates were incubated overnight at 35°C. Phage DNA was added to non-competent cells and plated similarly as a control.

Plasmid transformants were selected using the method of Youngman et al. (1985).

Plasmid DNA was transformed into *E. coli* cells using either the CaCl_2 treatment method described by Maniatis et al. (1982), or by electroporation (Dower et al. 1988). *E. coli* cultures (0.2 - 1.0 L) to be made 'electro-competent' were grown to early log-phase, centrifuged, washed in an equal volume of aqueous 1mM HEPES buffer (pH 7.0), concentrated tenfold, washed similarly in 1mM HEPES, then resuspended in 1/50-1/500 the original volume of 15% (v/v) sterile glycerol. The cells were dispensed in 0.5 ml volumes and frozen at -70°C until used.

Prior to pulsing, 25 μl of miniprep plasmid DNA (0.1-1.0 μg) was added to an 0.2 cm electrode gap width cuvette (Bio-Rad Laboratories, Inc., Richmond, CA) containing 375 μl thawed electro-competent cells and mixed by aspiration. The cuvette was chilled and pulsed with a Gene Pulser™ electroporator (Bio-Rad Laboratories, Inc., Richmond, CA) set at 2.5 KV (12.5 KV/cm), 25 μF ,

and 200 Ω resistance (pulse controller setting). Following the pulse the cells were immediately diluted with 1 ml S.O.C. broth (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) (Dower et al., 1988) and allowed to grow for 1 h in an additional 3 ml of broth at 37°C. Cells were plated on LB with the appropriate selective antibiotics.

Electroporation of *C. beijerinckii* NRRL B-592 was achieved as follows: static batch cultures were grown anaerobically to mid-log phase in 200 ml GC broth. The cells were placed in tightly capped 200 ml bottles, centrifuged at 15,000 X g for 10 min, washed with 20 ml HEPES electroporation buffer (HEB) (272 mM sucrose, 7 mM HEPES, 1 mM CaCl₂, pH 7.0) (Chassey and Flickinger, 1987), transferred to 45 ml tubes, centrifuged again at 12,100 X g for 10 min and finally resuspended in 2.5 ml HEB. Next, 0.75 ml cell samples were placed into ice-cold 0.4 cm electrode gap width cuvettes (Bio-Rad Laboratories Inc., Richmond, CA) containing 50 μ l plasmid DNA (0.1-1.0 μ g), and mixed well. A pulse of 2.5 KV (6.25 KV/cm) was applied at 25 μ F capacitance setting without using the pulse controller. The cells were removed from the hood, pulsed,

returned promptly after pulsing and 1 ml CBM medium containing 12.5 mM CaCl_2 and 12.5 mM MgCl_2 was rapidly added. The samples were further diluted tenfold and allowed to incubate for 2 h to allow time for expression of plasmid-borne genes, or for gene induction. Viable counts were done on cell samples taken both before and after the pulse was applied. After the expression period, 0.1 ml samples were plated directly and then the remaining culture was concentrated by centrifugation and plated on GC plates with appropriate selective antibiotics.

Continuous Culture Media

Slight modifications of Bott and Wilson's (1967) transformation medium were made to facilitate continuous culture experiments of *B. subtilis*. For nitrogen-limited chemostat experiments 3 mM $(\text{NH}_4)_2\text{SO}_4$ replaced all other N_2 sources. This medium was further modified for carbon/energy limited recycling fermentor experiments by increasing the $(\text{NH}_4)_2\text{SO}_4$ concentration to 0.2 % (excess) and reducing the amount of glucose to a limiting level of 4 mM.

Continuous culture devices and parameters

Chemostat cultures were used to grow *B. subtilis* at various

growth rates (mass doubling times, t_d) from the shortest possible t_d (μ max) to t_d near 10 h.

A Marubishi MD-250 2.5L aerobic fermentor (Enprotech Inc., Hyde Park, MA) was adapted to culture cells in chemostat and recycling fermentor modes (van Verseveld et al., 1984). In each case sterile medium was added to the growth vessel using a variable speed peristaltic pump (Rainin Instrument Co., Woburn, MA) and sterilized air was introduced by compression through a depth filter (Balston Inc., Lexington, MA) at a rate of 1L/min. A peristaltic pump (Cole-Parmer Instrument Co., Chicago, IL) was used to add medium to and abstract spent broth from the chemostat at equal rates. Steady state cell growth was established in the chemostat at each t_d by allowing at least 5 generations of growth before samples were taken.

Recycling fermentor mechanics have been described previously (Chesbro et al., 1979). The Marubishi fermentor was modified for use as an aerobic recycling fermentor as follows: a positive displacement pump (Fluid Metering, Inc. Oyster Bay, NY) was used to pump culture from the growth vessel across an Amicon TC1R tangential flow ultrafiltration apparatus (Amicon Inc., Danvers, MA)

fitted with a Nucleopore 0.2 μm poly-carbonate membrane filter (Nucleopore Corp. Pleasanton, CA). The recycling fermentor was first operated as a chemostat at a t_d of 1.5 h. When a steady state of growth was reached, the recycle pump was activated and the chemostat outflow port was closed. Cells were returned to the growth vessel while filtered medium was removed at a rate equal to the medium inflow rate. Thus there was 100% retention of biomass in the growth vessel while the medium input rate remained constant.

Dry weight determinations

Cell samples to be weighed were filtered or spotted onto 0.2 μm polycarbonate membrane filters (Nucleopore Inc., Pleasanton, CA). Bacterial dry weights were done according to the method of O'Toole (1983) and were weighed on a Cahn electrobalance (Cahn Division, Ventron Instruments Corp., Cerritos, CA).

Gas Chromatographic analysis

Cell samples were removed from the growth vessel, treated with formaldehyde (final concentration 0.1%), and stored for later analysis. Samples were clarified by centrifugation before the supernate was analyzed. A Perkin-Elmer 3920 (Perkin-Elmer, Norwalk, CT) gas chromatograph equipped with a flame ionization

detector and a glass column packed with 80/120 Carbopack B-DA 4% carbowax 20M (Supelco Inc., Bellefonte, PA) was used. One μ l of straight or diluted sample was injected and eluted isothermally at a temperature of 175°C. The nitrogen carrier gas flow rate was 24 ml/min. A volatile organic acids standard mix (Supelco Inc.) was used to calculate retention times and standard curves for quantitation.

BLM and BRM media formulation

BLM and BRM; *C. beijerinckii* L-colony, or Regeneration medium (Birrer et al., 1989), were prepared by the addition of stock solutions to a basal mixture. Stock solution A contained D-biotin, 1.0 g; PABA, 0.1 g; thiamine-HCL, 0.01 g; $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.1 g; $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$, 0.1 g; and $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 2.0 g, all in 100 ml H_2O . The solution was sterilized by filtration through a 0.2 μm membrane and maintained under a N_2/CO_2 atmosphere. Stock solution B consisted of 25 g glucose in 100 ml H_2O while stock solutions C and D consisted of 2.5 M solutions of MgCl_2 and CaCl_2 , respectively. Stock solution E contained 7.0 g of K_2HPO_4 and 3.0 g of KH_2PO_4 dissolved in 100 ml H_2O . Stock solutions B through E were autoclaved separately.

The basal mixture contained gelatin (Oxoid), 60.0 g; agar (Difco), 8.0 g; yeast extract (Difco), 8.0 g; casamino acids (Difco), 2.5 g; and L-asparagine, 1.0 g. These ingredients were mixed with either 930 ml H₂O for BRM or 910 ml H₂O for BLM and the mixture was stirred and brought to boiling before autoclaving at 121°C for 15 min. Upon cooling, 10 ml of stock solution A and 40 ml of solution B were added to the basal ingredients. In the preparation of BRM, 5 ml of each of solutions C and D were added while 15 ml of these solutions were added to prepare BLM. Stock solution E (10 ml) was added as the final component in preparing either BRM or BLM.

Protoplast Formation

Overnight cultures of *C. beijerinckii* NRRL B-592 grown to mid log-phase in CBM medium were diluted 1:4 with fresh, sterile CBM broth containing 0.4 % glycine (Allcock et al., 1982). When cultures became nearly 100 % motile as determined by phase-contrast microscopy, (45-60 min), the osmotic strength was increased by slowly adding a sucrose-lysozyme solution to final concentrations of 0.5 M and 2.0 mg/ml, respectively. After 5 min MgCl₂ and CaCl₂ were added to final concentrations of 12.5 mM each. Protoplast formation was complete after 60 min of treatment.

Growth of *C. bellerinckii* as L-colonies

Prior to plating, protoplast suspensions were diluted in CPM broth (CBM plus 0.5 M sucrose and 12.5 mM MgCl₂ and CaCl₂) (Allcock et al., 1982). In this medium the protoplasts remained intact for prolonged periods of time. Samples were plated onto BLM and L-colonies appeared after 4 d of incubation at 32°C. A relatively small number of bacillary colonies also appeared on some of the BLM plates. The numbers of these colonies usually correlated with the numbers of osmo-resistant forms determined by counting the number of colonies arising when protoplast samples were diluted in sterile, anaerobic H₂O and plated on non-osmotically reinforced media.

Transfer and regeneration of L-colonies on BRM

Large, well isolated L-colonies were picked and transferred to BRM plates for regeneration. L-colonies were transferred as agar plugs, removed with sterile Pasteur pipettes, inverted, and extruded onto marked sectors of BRM plates. These plates were incubated for 48 h or until regeneration occurred.

Purification of DNA and restriction endonuclease analysis

Ethanol-precipitated chromosomal or plasmid DNA was

dissolved in sterile glass-distilled H₂O. Ribonuclease A [E.C. 3.1.27.5] (RNase A, Sigma Chemical Co.) was added to a final concentration of 10 µg/ml, and the sample was incubated at 37°C for 30 min. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, Amresco, Inc.) was then added to the tube. This was gently shaken for 30 s and centrifuged at maximum speed in 1.5 ml polypropylene microcentrifuge tubes for 2 min. The upper aqueous layer was carefully aspirated and transferred to a second sterile tube. This process was repeated until there was no detectable protein layer at the interface and the sample was finally extracted with chloroform:isoamyl alcohol (24:1) to remove residual phenol. To avoid substantial loss of DNA during the extraction steps, additional H₂O was added to the remaining aqueous phase and extracted again. After ethanol precipitation the DNA was sufficiently clean for restriction analysis.

Restriction endonucleases were purchased from Gibco/BRL Life Technologies Inc. (Gaithersburg, MD) and were used according to the manufacturer's specifications except for the following modification. Incubation of the enzyme-DNA mixtures were carried out overnight at 4°C. Completeness of the digestion was verified by separating a

subsample by agarose gel electrophoresis and visualizing after ethidium bromide (EtBr) staining.

Agarose gel electrophoresis conditions, staining and photography

All plasmid DNA samples were separated by agarose gel electrophoresis on a horizontal mini-submarine apparatus (Bio-Rad Laboratories Inc., Richmond, CA) according to the methods outlined by Maniatis et al., (1982). Chromosomal DNA samples were run on a vertical electrophoresis apparatus (Hoeffer Scientific instruments Inc., San Fransisco, CA). When run at a constant voltage of 55 V, (approximately 30 mA) in tris acetate-EDTA buffer (0.4 M Trizma base, 0.2 M Na acetate, 20 mM EDTA, disodium salt), this system afforded better resolution of restriction fragments. Gels were soaked for 30 min in a 1 µg/ml solution of EtBr and placed on a U.V. light transilluminator for visualization and photography.

Gels were photographed with a polaroid system using Type 55 positive/negative film in combination with a Kodak gelatin filter #16. The usual exposure at F-stop setting 4.7 and the shutter control on B(ulb) was 2.5 min.

Nick translation of probe DNA

DNA was labelled with ^{32}P by nick translation. Nick translation kits (containing DNA Pol I enzyme, DNase I enzyme, and various deoxynucleotide mixtures) purchased from Gibco BRL, Life Technologies, Inc., (Gaithersburg, MD) were used. d CTP, or d GTP (α - ^{32}P) was purchased from Du Pont Company (New England Nuclear Research Products, Boston MA). The labelling process was carried out *in vitro* by the nick translation method described by Maniatis et al. (1982) and according to the manufacturers instructions. Fully labelled probe DNA was separated from unincorporated nucleotides by 2 sequential ethanol precipitations.

DNA dot-blot hybridization

The procedure used here for probing spotted DNA samples on nitrocellulose filters was adapted from a colony and plaque hybridization procedure distributed by Gelman Sciences (Ann Arbor, MI). DNA samples to be probed were applied to Biotrace™ NT nitrocellulose binding matrices (Gelman Sciences, Ann Arbor, MI) by spotting 10 μl volumes containing approximately 100 μg of DNA. The positions of the spots were marked on a template of the membrane for future reference. The filter was soaked in a tray containing absorbent filter paper saturated with DNA denaturing solution (0.5 M

NaOH, 1.5 M NaCl) for 2 min, removed, and placed in a similarly prepared tray of neutralizing solution (1.0 M Tris-HCL, 1.5 M NaCl). After 5 min, the filter was rinsed thoroughly with a squirt bottle containing 2X SSC (0.3 M NaCl, 0.03 M Na-citrate) to remove all debris from the membrane. It was then air dried and incubated in a vacuum oven at 80°C for 1 h. Next the filter was sealed in a plastic bag with enough BEPS buffer (1 % bovine serum albumin, [BSA], 1 mM disodium EDTA, 0.5 M Na-phosphate, [pH7.2], 7 % SDS) to allow uninhibited movement of the filter(s) in the bag (5-10 ml per membrane). The filter was prehybridized by incubating with shaking (if available) for 10 min at 65°C. The BEPS buffer was decanted and the same amount of fresh BEPS buffer containing the probe ($\sim 2\text{-}4 \times 10^5$ CPM per membrane) was carefully added to avoid bubble formation in the bag. The probe DNA was denatured by heating to 100°C for 5 min and quickly chilling in ice water before addition. The DNA was allowed to hybridize overnight at 65°C with shaking (if available). The filter was then extensively washed to remove any residual nonspecifically bound probe. The hybridization solution was carefully decanted from the bag and either saved for reuse or disposed of properly. The membrane was then washed in wash

solution I (WSI) (0.5 % BSA, 1 mM disodium EDTA, 40 mM Na-phosphate, [pH 7.2], 5 % SDS) at 65°C in the following sequence: 50 ml for 5 min in the bag, 200-500 ml for 20 min in a large tray, 200-500 ml for 10 min. The filter was washed in WSII at 65°C in the following sequence: 200-500 ml for 20 min, 200-500 ml for 20 min, 200-500 ml for 20 min. The filter(s) were removed from the tray, air dried, and mounted for autoradiography.

Southern transfer and hybridization

The method of Southern (1975) was used to transfer chromosomal DNA fragments from agarose gels to nitrocellulose filters for subsequent hybridization with probes. The transfer procedure was essentially the same as the capillary action procedure described in Maniatis et al. (1982). Transfer was allowed to continue for 24 h after which the DNA was captured on a Biotrace™ NT nitrocellulose filter (Gelman Sciences, Ann Arbor, MI).

DNA quantitation

Three methods were used to quantify various purified DNA samples during the course of this study. When large amounts of chromosomal DNA were obtained from *B. subtilis*, the diphenylamine method of Burton (1956) was used. When plasmid DNA samples of

relatively low concentration were quantitated, the ethidium bromide dot method of Selden and Chory (1988) was used. This method involved the visual discernation of the intensity of ethidium bromide-stained dots of test DNA as compared to a set of standards.

Standards were made by diluting pure phage λ DNA to concentrations of 1.0, 2.5, 5.0, 7.5, and 10 $\mu\text{g/ml}$. Each standard, including serially diluted test DNA samples, was combined with an equal volume of a 1 mg/ml solution of EtBr. Then 8.0 μl of the samples were spotted onto plastic wrap that has been spread over the lens of a U. V. transilluminator. The concentration was determined by visually gauging the intensity of the test spots relative to the standards.

The third method used to quantitate DNA samples after extensive purification was U. V. light absorption at a wavelength of 260 nm. This method has been described in detail elsewhere (Maniatis et al., 1982).

III. RESULTS

Natural transformation in *Bacillus subtilis*: development of the competent state

Competence for transformation involves the passage of a *Bacillus subtilis* culture to a distinct physiological state characterized by the ability to bind and incorporate exogenous DNA sequences. This state develops when the cells are grown in glucose-minimal salts media (not in complex media such as nutrient broth or LB). Only 10-20% of the cells in a given population ever become competent (Wilson and Bott, 1968). The onset of competence has been shown to be accompanied by a relative metabolic latency involving a decrease in DNA synthesis (Dooley et al., 1971; Dubnau, 1982). Competence has been described in the literature as occurring in batch culture during the early stationary phase when cell growth has largely ceased (Dubnau, 1982). It has also been shown to occur at rather rapid (and balanced) growth rates such as those attainable in chemostat culture (Portoles et al., 1977). An initial purpose of the studies presented here was to examine the relationship between

growth rate, cell metabolism and competence in *B. subtilis* in an attempt to clarify conflicting perceptions of the culture's growth rate at the time of competence expression.

The development of competent *B. subtilis* at various growth rates in chemostat culture

The relationship between growth rate and the development of competence in *B. subtilis* was examined. Steady-state chemostat cultures of *B. subtilis* 168 wt or *trpC2* were established at various growth rates corresponding to mass doubling times (t_d) between 0.75 and 10.0 h in a glucose minimal salts transformation medium (Bott and Wilson, 1967). When cell samples were assayed for transformation the highest numbers and largest percentage of competent cells developed in the growth vessel at a t_d of about 2.5 h (Fig. 1). The culture was observed to progress from a low level of competence at the more rapid t_d range between 0.75 and 2.0 h, peak at 2.5 and become relatively non-transformable at the less rapid steady states established between t_d of 4 and 7 h (Fig. 1). This pattern was also observed with plasmid DNA (pTV32ts, Youngman et al., 1985), or when the wild type strain was grown under nitrogen limitation and bacteriophage 41C DNA (Zsigray et al., 1973) was

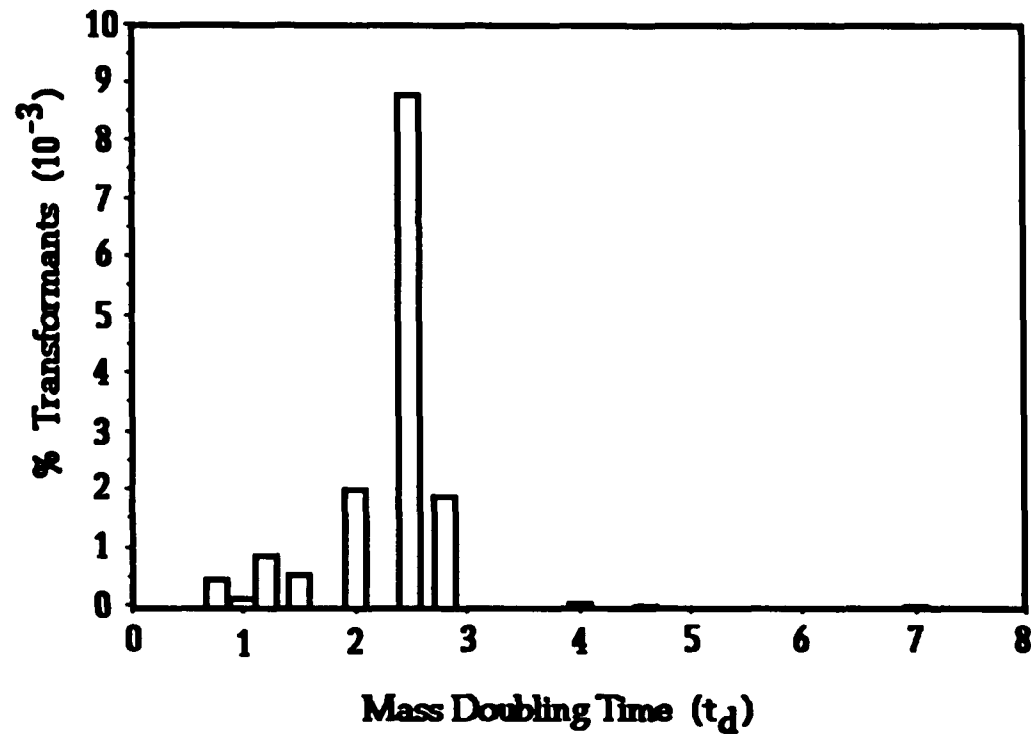


Figure 1. Percentage of *B. subtilis* trp C2 population transformable to prototrophy after being removed from chemostat cultures established in steady state growth at various rates and incubated with wild-type chromosomal DNA. Each bar represents an average of at least three determinations from the corresponding growth rate.

used to measure transformation.

Relationship between steady-state biomass concentration and the development of competence in chemostat cultures

Steady state biomass yields (X , mg/ml), using dry weight measurements of the chemostat cultures grown at various rates, were calculated. X describes the total amount of biomass that exists in the growth vessel at a particular steady state. Figure 2 shows steady state biomass yield values plotted as a function of growth rate (t_d). The biomass yields were observed to change in a growth rate-dependent manner. An inflection in the curve was observed at a t_d of 2.5 h, the t_d of maximal competence (Fig. 2). At the faster growth rates (< 2.5 h) X was low. The lowest values occurred nearest the maximal growth rate (t_d max) then gradually increased to the highest values near a t_d of 2.5 h. At less rapid growth rates (beyond 2.5 h, out to 7.0 h) the yield slightly decreased (Fig. 2).

Search for competence at ultra-slow growth rates and spill metabolism of *B. subtilis* cultures expressing competence

Studies in the literature suggested that competence occurred at relatively slow growth rates, when growth sustaining nutrients

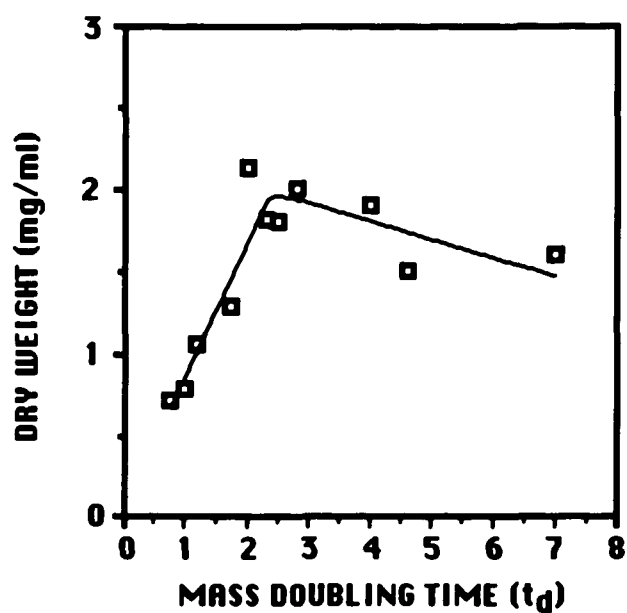


Figure 2. Steady state biomass concentrations (X , mg/ml) plotted as a function of growth rate (t_d) of *B. subtilis* grown under nitrogen limitation in chemostat cultures. Each point represents an average of at least three samples taken at each steady state growth rate.

become exhausted from the medium (Anagnostopoulos and Spizizen, 1960; Dubnau, 1982). Therefore *B. subtilis trp* C2 was grown in a biomass recycling fermentor in order to scan the largest possible range of growth rates for any other competence events and to examine other cellular processes at the t_d of competence development. Since homogeneity of t_d in a population growing in a chemostat, as usually operated, is progressively lost at doubling times longer than 10 h (Hansford and Humphrey, 1966), a continuous biomass recycling fermentor was needed to expand this growth rate range by about a factor of 10. Carbon/energy limited bacterial cultures growing at t_d from within the chemostat range (< 10 h) to doubling times in excess of 100 h have been routinely obtained with this growth system (Chesbro et al., 1979; Chesbro, 1988). This range extends from the fastest achievable in a minimal medium to ultra slow rates difficult to examine in any growth system other than fed-batch cultures because of their fleeting temporal existence. These ultra slow rates are roughly comparable to those of the stationary phase of batch-type bacterial growth, only they are prolonged and are more homogenous with respect to cellular metabolism during slow growth (van Verseveld et al., 1984). Figure

3 shows the increase in optical density of a carbon/energy limited (4 mM glucose), recycled culture of *B. subtilis* with time. As the cell density in the reactor increases (because the cells are returned to the growth vessel as spent medium is removed through a 0.2 μm filter) the mean growth rate steadily falls due to a gradually decreasing concentration of limiting nutrient per cell. Cell samples were periodically tested for competence by transformation of the *trpC2* strain to prototrophy. The kinetics of transformable cells arising in the culture as the growth rate decreases is shown in Fig. 3. The number of transformable cells began to increase 8 h into the run, when the growth rate of the culture was calculated to be approximately 2.5 h (t_d). No additional competence events were detected at any later time.

Samples were removed from the growth vessel at regular intervals, centrifuged, and the supernate analyzed for organic acids by gas chromatography. Acetate was found to be the predominant product under these growth conditions. Figure 3 also shows the amount of acetate produced by *B. subtilis* in the recycling fermentor. The culture produced the largest amount of acetate during rapid growth, which averaged 3.5 mM (this is a steady-state level,

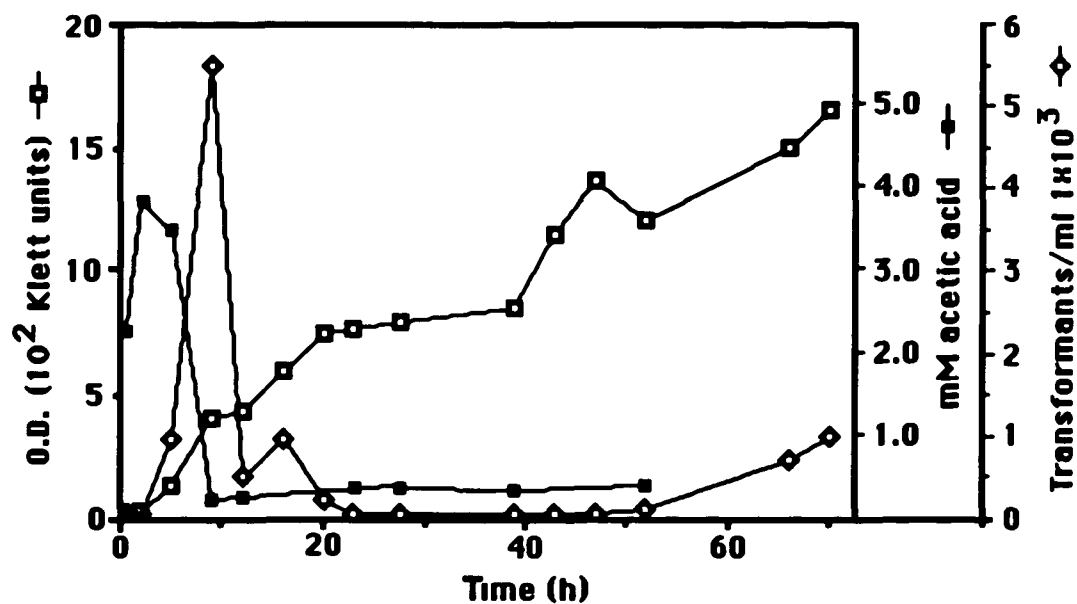


Figure 3. Plot showing the increase in O.D., numbers of competent cells and levels of acetate formed by *B. subtilis* grown under carbon/energy limitation in a biomass recycling fermentor.

because the acetate is continuously removed from the growth vessel with the medium). It then decreased tenfold to 0.3 mM about 8 h into the run, as the growth rate fell below a t_d of 2.5 h, and remained at this lower level. It can be seen from Fig. 3 that the timing of this event coincides precisely with the development of competence in the culture. The culture virtually ceased excreting acetate as the growth rate fell past a t_d of 2.5 h, the growth rate of competence expression in *B. subtilis*.

Sporulation of *B. subtilis* in a biomass recycling fermentor culture and its relationship to competence development

The occurrence of phase bright spores in the recycled culture is shown in Fig. 4. Sporulating cells were present in the culture throughout the fermentation. A low number of spores were first detected when the culture was in chemostat mode. Numbers of phase-bright forespores, phase-dark and free spores were first detected at a percentage greater than 1 % of the total cells 13.5 h into the run. This corresponds to just after the observed peak in competent cells. No single, massive sporulation event was detected at any point in the recycled culture. Rather, a continuous increase of cell types in progressively later stages of sporulation was observed

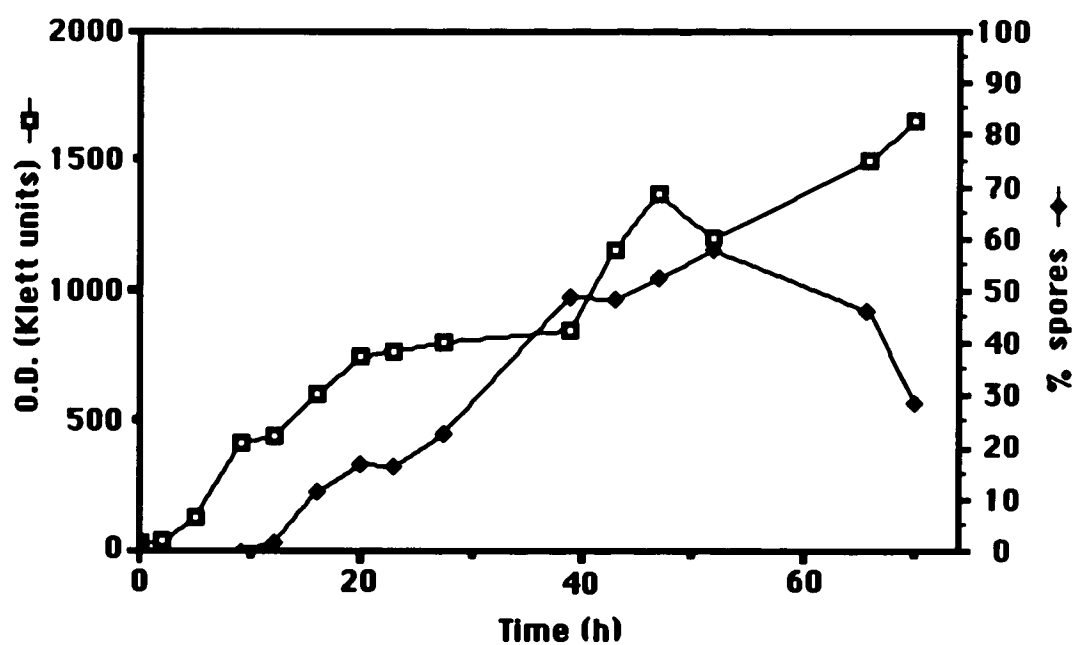


Figure 4. Optical density and percentage of spores plotted as a function of time of the growth of *B. subtilis* under carbon/energy limitation in a biomass recycled culture. % spores includes phase bright, phase dark and free spores.

in the vessel as the fermentation proceeded. Generally, the amount of sporulating cells varied inversely with the growth rate of the culture.

Development of a protoplast manipulation system for *C. beijerinckii* : protoplast formation of *C. beijerinckii*

The information gained from the physiology of *B. subtilis* cells as they attain competence was applied to *Clostridium beijerinckii* NRRL B-592. Attempts to render *C. beijerinckii* naturally competent for transformation with plasmid DNA of Gram positive bacterial origin (Table 2) were unsuccessful. Transformants could not be obtained after cultures were prepared in the manner developed by Anagnostopoulis and Spizizen (1960) to render *B. subtilis* competent, or when treated with CaCl_2 according to the method for *E. coli* transformation described in Maniatis et al. (1982). Likewise, conjugation experiments failed to produce positive results. In light of this and because reports of success with genetic transfer systems based on protoplast manipulation of other bacteria such as *B. subtilis* and *Streptomyces* were encouraging, a gene transfer system for *C. beijerinckii* involving protoplasts was sought.

Maximum conversion of bacillary forms to protoplasts

occurred when rapidly growing, exponential phase cultures of *C. beijerinckii* NRRL B-592 or B-593 were treated with 2 mg/ml lysozyme in CBM broth (O'Brien and Morris, 1971) containing 0.5 M sucrose as osmotic stabilizer. The degree of lysozyme sensitivity varied directly with the motility of the cells. Protoplast formation was either absent or greatly reduced in overnight cultures or in any cultures containing clostridial forms or phase-bright forespores. Cultures whose growth rates were upshifted by a fourfold dilution in fresh CBM broth containing 0.4 % glycine (Allcock et al., 1982) became increasingly susceptible to lysozyme treatment until 1h after upshift, when > 99 % wall-less forms could be obtained. Post-upshift times longer than 1 h resulted in lower protoplast yields. Protoplasts were observed extruding from the sides or poles of the cells.

Formulation of L-colony and regeneration media for *C.*

***beijerinckii* NRRL B-592**

A comparison of the regeneration media devised for strains of *C. acetobutylicum* by Allcock et al. (1982) and Reilly and Rogers (1987) showed that the concentrations of agar, gelatin, CaCl_2 and MgCl_2 varied considerably in these media and were most likely

affecting regeneration. Both media were based on CBM. The former solid medium contained 2 % agar and 5 % gelatin, and the latter contained 0.8 % and 0 %, respectively. Both media contained high concentrations, although different, of calcium and magnesium in the form of their divalent chloride salts. Therefore, due to wide discrepancies observed in these critical medium ingredients, a Box-Behnken 3-parameter optimization strategy (Greasham and Inamine, 1986) was used to test the effect of various concentrations of these ingredients on regeneration and L-colony growth of *C. beijerinckii* NRRL B-592. Two distinct solid media were formulated. The first supported growth of L-colonies (BLM), and the second permitted regeneration of the L-colonies (BRM). Figure 5A shows a typical L-colony (arrow) growing next to a bacillary colony of *C. beijerinckii* B-592 on a BLM plate. Some bacillary colonies appeared on BLM plates, but because the number of these correlated with counts of osmotically resistant cells, they were assumed to have arisen from lysozyme resistant bacteria. B-592 protoplasts plated on BLM routinely grew as large L-colonies (1 mm in diameter) and at frequencies ranging between 2.4 and 5.5 % of the total number of protoplasts (Table 7). When protoplasts were

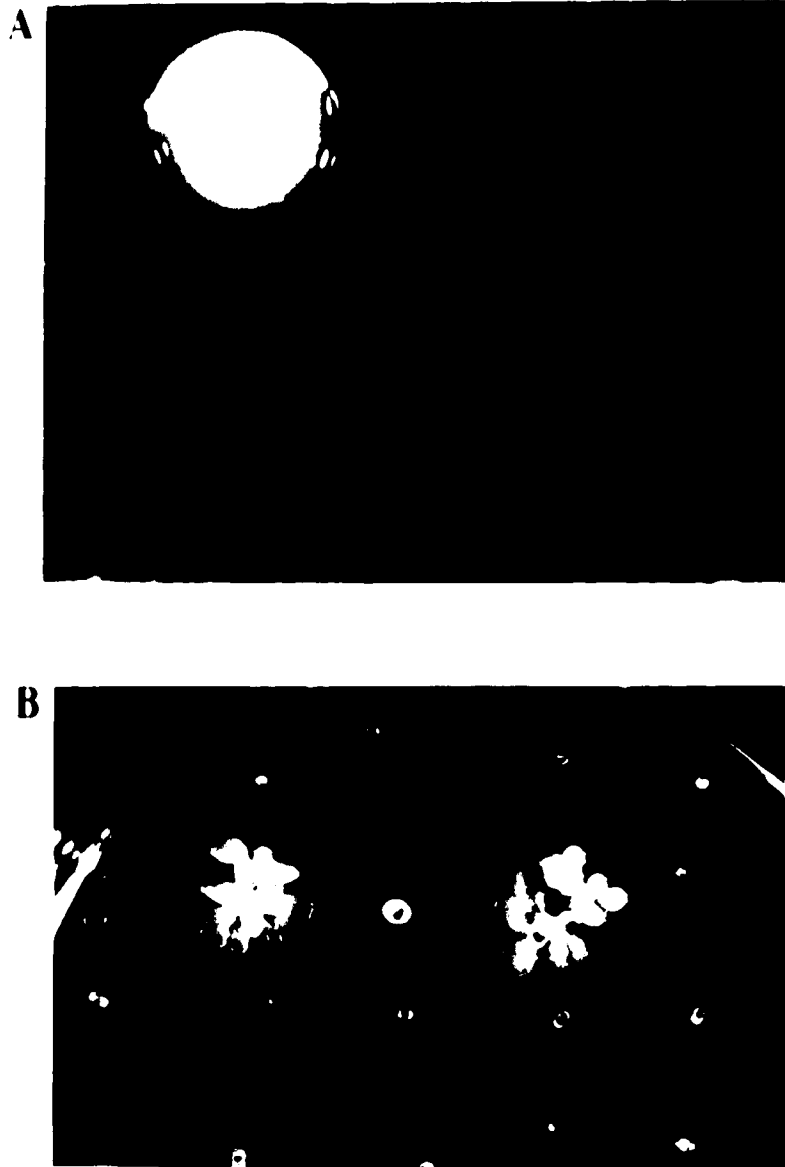


Figure 5. Photograph A.; *Clostridium beijerinckii* B-592 growing on solid BLM medium. A typical L-colony (arrow) is shown growing next to a bacillary colony. Photograph B. shows three L-colonies that have reverted to the bacillary state after being transferred from a BLM plate and allowed to grow for three days on a BRM plate.

TABLE 7. Growth of *C. beijerinckii* NRRL B-592 as L-colonies on BLM medium

	VIABLE CELLS ^a	OSMO-RESISTANT ^b	L-COLONY ^c	% L-COLONY ^d
EXP. #	CFU/ML	CFU/ML	CFU/ML	CFU/ML
1	8.6 X 10 ⁶	1.0 X 10 ³	4.1 X 10 ⁵	4.8
2	1.1 X 10 ⁷	5.3 X 10 ³	6.0 X 10 ⁵	5.5
3	5.1 X 10 ⁶	< 10	1.2 X 10 ⁵	2.4
MEAN:	8.2 X 10 ⁶	2.1 X 10 ³	3.8 X 10 ⁵	4.2 %

^a Viable cells prior to addition of 2 mg/ml lysozyme.

^b Lysozyme-treated samples diluted 1:10 in anaerobic H₂O and plated on CBM agar plates.

^c Number of L-colonies appearing after 6 d incubation on BLM plates.

^d a/c X 100.

TABLE 8. Regeneration of L-colonies to bacillary colonies.

EXP #	# L-COLONIES TRANSFERRED	# L-COLONIES REGENERATED	% REGENERATION
1	114	56	49
2	90	8	9
3	77	12	16

Average % regeneration from L-colonies = 25

plated directly onto BRM regeneration plates the frequencies of L-colony formation and regeneration were lower than when protoplasts were first plated on BLM, grown as L-colonies, and then transferred to BRM. The data in Table 8 show that the frequency of regeneration of transferred B-592 L-colonies ranged between 9 and 49 % and that the average final regeneration frequency was 25 %. Figure 5B is a photograph of a BRM plate onto which L-colonies had been transferred. It illustrates the drastic change in colonial morphology that accompanies regeneration of an L-colony. The large spreading colonies are bacillary colonies of B-592.

L-colony growth and regeneration of other solventogenic clostridia on BLM and BRM

Three other solventogenic clostridial strains were examined for their ability to grow as L-colonies on BLM and to regenerate on BRM. Protoplasts of *C. beijerinckii* NRRL B-593, and *C. acetobutylicum* strains ATCC 10132 and ATCC 4259 could be produced by the method developed for B-592. Table 9 shows the B-593 strain of *C. beijerinckii* and the 10132 strain of *C. acetobutylicum* grew as L-colonies and regenerated to bacillary colonies when L-colonies were transferred to BRM medium. Strain

**TABLE 9. L-colony growth and regeneration of L-colonies
of other solventogenic *Clostridium* species.**

ORGANISM	% L-COLONIES	% REGENERATION
<hr/>		
<i>C. beijerinckii</i> NRRL B-593	0.23	6.9
<i>C. acetobutylicum</i> ATCC 10132	4.1	8.6
<i>C. acetobutylicum</i> ATCC 4259	---	---

% L-colonies calculated as follows:

L-colonies divided by the # viable cells before lysozyme
addition minus the osmo-resistant forms times 100.

10132 formed L-colonies at a frequency of 4.1 % and its transferred L-colonies regenerated at a frequency of 8.6 %. Strain B-593 formed L-colonies on BLM at a frequency of 0.23% of the number of cells protoplasted. The L-colonies regenerated 7% of the time when transferred to BRM. *C. acetobutylicum* strain ATCC 4259 did not grow as either form on these media.

Analysis of PEG-mediated protoplast transformation and protoplast fusion as methods for genetic recombination in

***C. beijerinckii* NRRL B-592**

Using the protoplast formation/regeneration system described above, PEG-induced protoplast transformation of *C. beijerinckii* with plasmid DNA was examined. *C. beijerinckii* protoplasts were treated with 40 % PEG 6000 in the presence of plasmid DNA from various Gram positive sources (Table 2). Protoplast or bacillary colonies expressing the appropriate marker genes after protoplast transformation with plasmid DNA from *B. subtilis* (pIM13), *S. aureus* (pTV1,8,32,53, pC194, pE194 and pUB110), *B. cereus* (pBC16) and *B. anthracis* (pLS20), were not detected.

The protoplast fusion method reported by Allcock et al. (1982) for *C. acetobutylicum* was then evaluated using *C. beijerinckii* NRRL

B-592. The mesophilic, cellulolytic *Clostridium* strain C7 was chosen to be fused with *C. beijerinckii* mainly due to its ability to degrade cellulose. The β -glucanase enzymes secreted by strain C7 facilitated screening for recombinant organisms because they were useful as markers and for selective purposes. Fusion was attempted by centrifuging various ratios of both types of protoplasts in a single tube and resuspending the pellet with PEG according to the method of Jones et al. (1985). After several wash steps to remove the moderately toxic PEG the protoplasts were plated on *Beijerinckii* L-colony medium (BLM) to grow the potential recombinants as L-colonies. Well isolated L-colonies arising on the highest dilution plates were chosen and aseptically transferred to BRM plates for regeneration. Regenerated colonies were streaked for isolation on GC plates and examined for expression of the desirable traits of the two parent strains, namely; cellulolysis including endoglucanase and exoglucanase enzyme activity (from C7), starch hydrolysis (from *C. beijerinckii*), and the characteristic acids and solvents produced by *C. beijerinckii*. In this manner pure cultures were obtained that showed a single colony type that was in most cases different from either parent. The cells stained Gram positive, and were anaerobic

bacilli. Three general types of putative fusants were found after phenotypic analysis. Types I and II produced and secreted amylase as well as showed carboxy methyl cellulase (CMCase) activity, produced an intracellular exoglucanase enzyme activity (which was only detectable after permeablizing the cells with chloroform vapor) and produced acetate, ethanol and acetone as fermentation products from glucose. The greatest percentage of fusants were type I which differed from type II by a lack of butyric acid production. The type III isolate had a colonial morphology and fermentation product profile identical to the parent *C. beijerinckii* NRRL B-592. Genomic DNA was isolated from a typical (type I) and an atypical (type III) fusant. The thermal melting points of these and the parent strains were determined in order to calculate and compare the guanosine plus cytosine (G+C) ratios. The G+C ratio of the fusants was found to be 51 ± 2 , the ratios of *C. beijerinckii* and C7 were found to be 33 ± 2 , and 39 ± 2 , respectively. Since mole percent G+C alone is insufficient to unequivocally determine the relatedness of the fusant to the parents, an S1 nuclease DNA-DNA liquid hybridization study was undertaken to assess the degree of sequence homology between the DNA of the parents and that of a fusant strain. Table 10

shows normalized % homologies of the various DNA combinations. It was found that the DNA of one of the fusants that showed a typical combination of desirable traits did not show a greater additive % homology to the parents than the parents showed to each other. Therefore, after a considerable effort to demonstrate that protoplast fusion of these bacterial strains was occurring and was a feasible genetic recombination system for *C. beijerinckii*, the results were largely unsatisfactory.

Electro-transformation of *Clostridium beijerinckii* NRRL B-592 with plasmid DNA

An electricity-mediated gene exchange system was next evaluated by testing electroporation as a way to introduce plasmid DNA to *C. beijerinckii*. The widely used *S. aureus*-*B. subtilis* vectors shown in Table 2, as well as vectors from *B. cereus* (pBC16) and *B. anthracis* (pLS20), were not observed to be expressed in *C. beijerinckii* B-592 after electroporation. Of the plasmids shown in Table 2, only pHR106 and plasmids later derived from this replicon were transferable to *C. beijerinckii* by this method. The Gene Pulser™ electroporation apparatus was used to transfer CCC pHR106 DNA dissolved in electroporation buffer to untreated cells of *C.*

Table 10. Normalized DNA-DNA homologies of *C. beijerinckii* B-592, *Clostridium* strain C7 and Fusant F4

Experiment #	Hybridization combination	%Homology	average % Homology
1	592*/C7	56.0	33.8
2	592*/C7	11.5	
1	C7*/592	32.0	29.0
2	C7*/592	26.0	
1	F4*/592	36.0	30.5
2	F4*/592	25.0	
1	592*/F4	14.6	10.8
2	592*/F4	7.2	
1	C7*/F4	47.0	29.0
2	C7*/F4	11.0	
1	F4*/C7	28.0	23.8
2	F4*/C7	7.0	

* Denotes labelled DNA sample.

beijerinckii NRRL B-592. Colonies expressing the chloramphenicol (Cm) resistance gene of pHR106 (Roberts et al., 1988; Abraham and Rood, 1987) were observed on selective GC plates containing 25 µg/ml Cm after *C. beijerinckii* cells were subjected to a 2.5 KV electrical pulse (6.25 KV/cm) applied at a capacitance of 25 µF without using the "pulse controller" (a set of resistors in series) supplied by the manufacturer. The frequency of transfer of *Cm^r* based upon the number of cells surviving the electrical pulse was about 1×10^{-7} . None of the *Cm^r* transformants were found to express the ampicillin resistance gene (β -lactamase gene from pBR322) carried by pHR106. Cytoplasmic plasmid DNA could not be visualized by agarose gel electrophoresis after extraction from the *Cm^r* transformants. However, plasmid DNA was visualized after simultaneously performed control extractions from a strain of *C. perfringens* (ATCC 12505) known to contain three plasmids.

Confirmation of chromosomal insertion of pHR106 in *C.*

beijerinckii

Electroporation of *C. beijerinckii* with pHR106 reproducibly resulted in *Cm^r* colonies, but no autonomously replicating plasmid DNA could be detected in the transformants. Therefore, it was

necessary to examine if the plasmid had integrated into the chromosome. Chromosomal DNA samples from the wild-type *C. beijerinckii* NRRL B-592, *Cmr* transformant C14, *B. subtilis* 168, and plasmid pHR106 DNA were spotted on a nylon filter and probed with ³²P-labelled pHR106 DNA in a dot-blot test. Figure 6 shows the autoradiogram of this dot-blot test. Hybridization of pHR106 probe DNA was detected to the pHR106 spot (positive control) and the *Cmr* transformant C14 spot. No detectable hybridization of pHR106 to the wild-type *C. beijerinckii* DNA or to *B. subtilis* DNA occurred.

To more precisely characterize the nature of the integration of pHR106 into the genome of *C. beijerinckii*, Southern blots of *Xba* I digested chromosomal DNA from both the wild-type and *Cmr* transformants C1-6, and C14, separated by agarose gel electrophoresis (Fig. 7A) were probed with ³²P labelled pHR106 DNA. While the *C. beijerinckii* chromosome contains many cut sites for *Xba* I, pHR106 was found to contain no cut sites for this enzyme. The autoradiogram shown in Fig. 7B revealed pHR106 DNA hybridized to a 14.3 Kb fragment of transformant DNA (arrow, lanes 4 through 10). No hybridization was seen to the wild-type DNA shown in Fig. 7B, lanes 1 and 3. The same band in each of the 7 transformant DNA

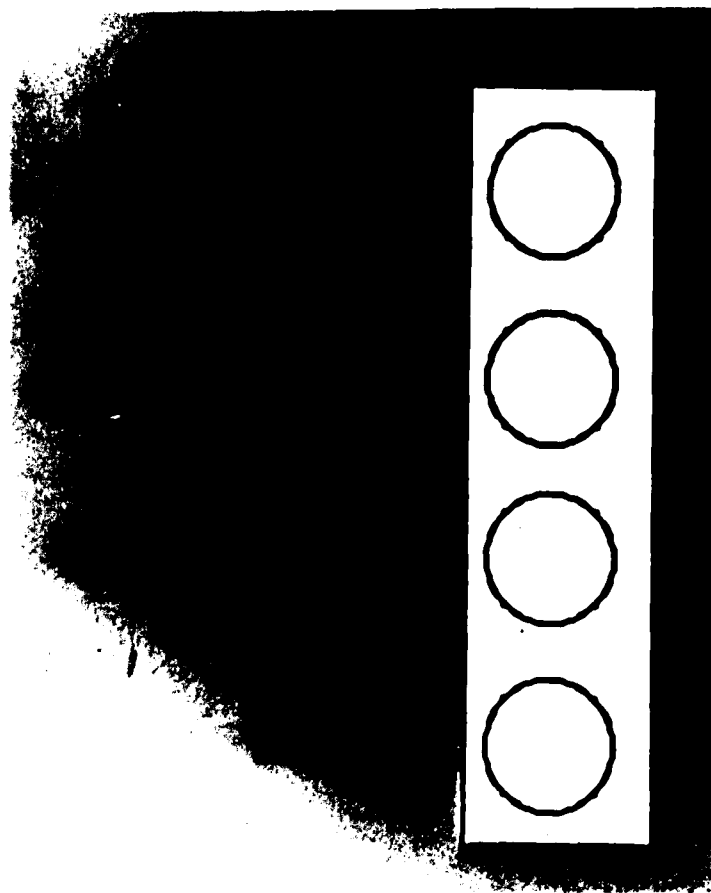


Figure 6. Dot-blot hybridization test in which 10 μg samples of chromosomal DNA were spotted as indicated onto a nitrocellulos filter, denatured, and probed with denatured ^{32}P - labelled pHR106 DNA.

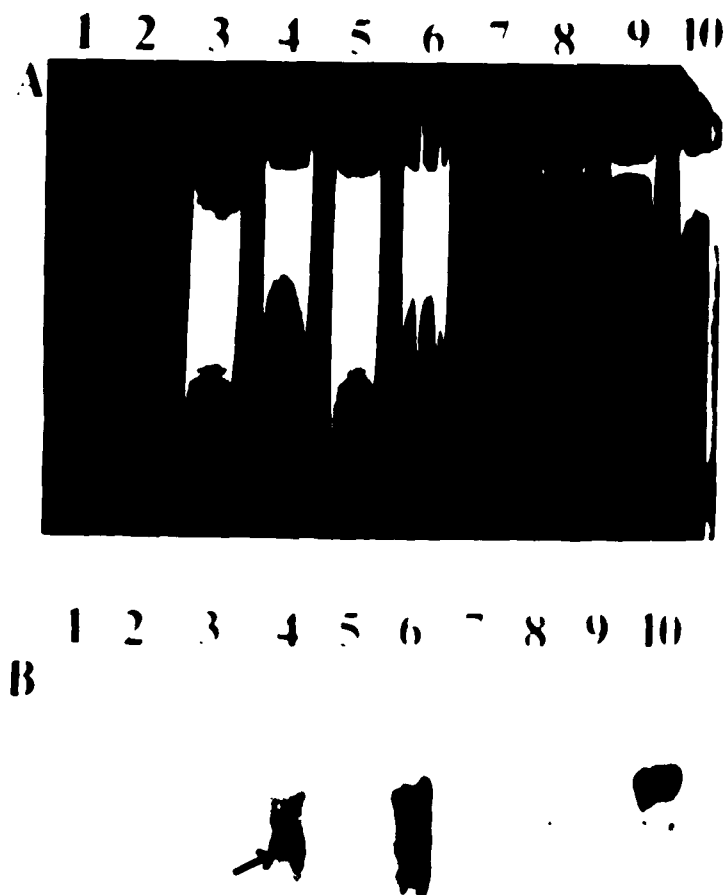


Figure 7. Agarose gel of *Xba* I digested chromosomal DNA samples (A), and Southern transfer of same gel probed with ^{32}P -labelled pHR106 DNA (B). Lanes a and c contain *C. beijerinckii* B-592 wild-type DNA. Lanes 4-10 contain DNA extracted from seven separate Cm^r isolates. The arrow is pointing to the 14.3 kb band containing pHR106 sequences.

samples, except that of lane 7, which was degraded into fragments smaller than 14 Kb, showed the strongest hybridization to pHR106. Autoradiograph bands corresponding to the highest MW DNA (Figure 7B) represent DNA that was incompletely digested by Xba I. Figure 8A is a photograph of two agarose gels of Xba I digested chromosomal DNA from the wild-type *C. beijerinckii* and Cmr transformants including various plasmids added as positive controls. Figure 8B shows photographs of the corresponding autoradiographs of Southern transfers of these gels, showing, on the left side, hybridization of ³²P labelled pHR106 to pBR322 DNA and pHR106 DNA added as positive controls (pBR322 sequences are present in pHR106). Shown also in lanes 3, 7, and 8 are Xba I digested chromosomal DNA samples from Cmr transformants. Similarly treated chromosomal DNA from the wild-type is shown in lane 4 (no hybridization under these conditions). The autoradiogram on the right side of Fig. 8 (lanes 8-11) shows pBR322 and pHR106 DNA samples in lanes 8 and 9, respectively, Xba I digested chromosomal DNA samples from wild-type *C. beijerinckii* in lane 10 and C14 transformant DNA in lane 11. When this was probed with ³²P labelled pBR322 DNA it hybridized to the same 14.3 Kb band (lane

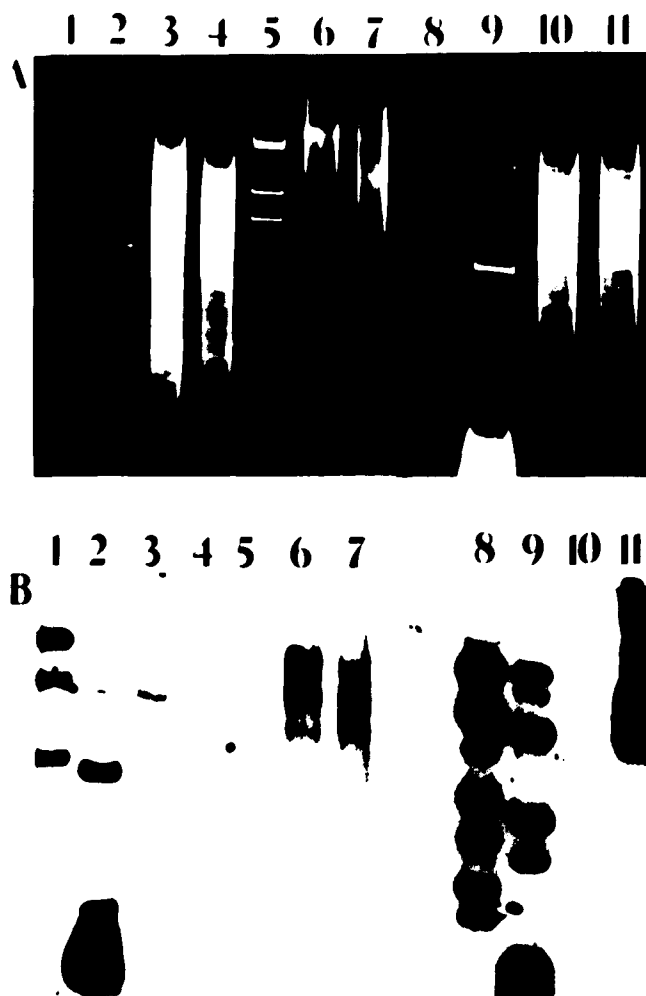


Figure 8. Agarose gels (A), and corresponding Southern transfers (B), probed with either pHR106 (lanes 1-7) or pBR322 (lanes 8-11). Lanes 1 + 8 and 2 + 9 contain pBR322 and pHR106, respectively. Lanes 3 and 11 contain Xba I digested chromosomal DNA from *Cm^r* transformant C14. Lanes 6 and 7 contain similarly treated DNA from two other *Cm^r* transformants of *C. beijerinckii*. Lane 5 contains bacteriophage lambda HindIII restriction fragments. The sizes of these bands are; 22.3, 9.4, 6.4, 6.2, 4.4, and 4.2 kb.

11). These data collectively show that pHR106 integrates into the *C. beijerinckii* chromosome apparently in a non-random fashion and integrates along with sequences that are not expressed in the transformants.

Can chromosomally integrated pHR106 sequences direct the integration of other homologous plasmids?

Since there was a strong possibility that pHR106 integrated into the *C. beijerinckii* chromosome by homologous recombination, other plasmids sharing sequence homology with pHR106 were examined for their ability to integrate into a strain already containing integral pHR106 sequences. Plasmid pHR106 consists of a substantial portion of the *E. coli* cloning vector pBR322 shown blackened in the drawing of pHR106 in Fig. 9. Two plasmids, pJIR71 (pUC18 Ω Pst I:pJIR39, 2.9 Kb Tc [Abraham et al., 1988]) and pJIR229 (pUC18 Ω Pst I-Bal31:pJIR208, 1.0 Kb MLS [Berryman and Rood, 1989]), were obtained that contain clostridial antibiotic resistance genes cloned into pUC18. The maps of these two plasmids are shown in Figure 10 (pUC18 sequences are shown blackened). Plasmid pJIR71 contains a 2.9 Kb fragment from *C. perfringens* that confers tetracycline (Tc) resistance, *tetP*, and pJIR229 contains a 1.0 Kb

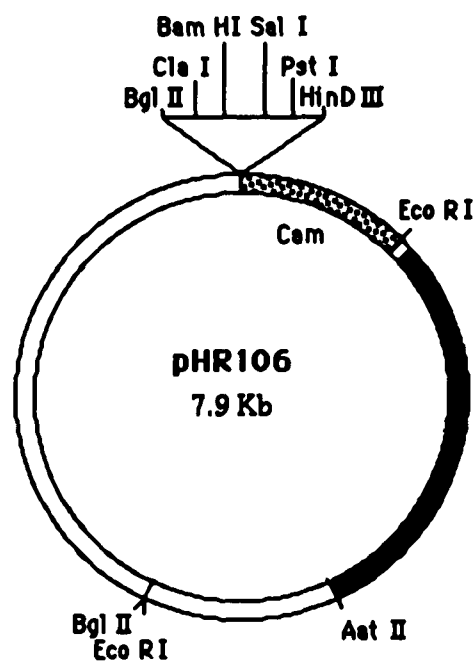


Figure 9. Restriction map of pHR106 (Roberts et al., 1988). The blackened area represents pBR322 (or pUC18) sequences. The stippled area denotes the chloramphenicol resistance gene.

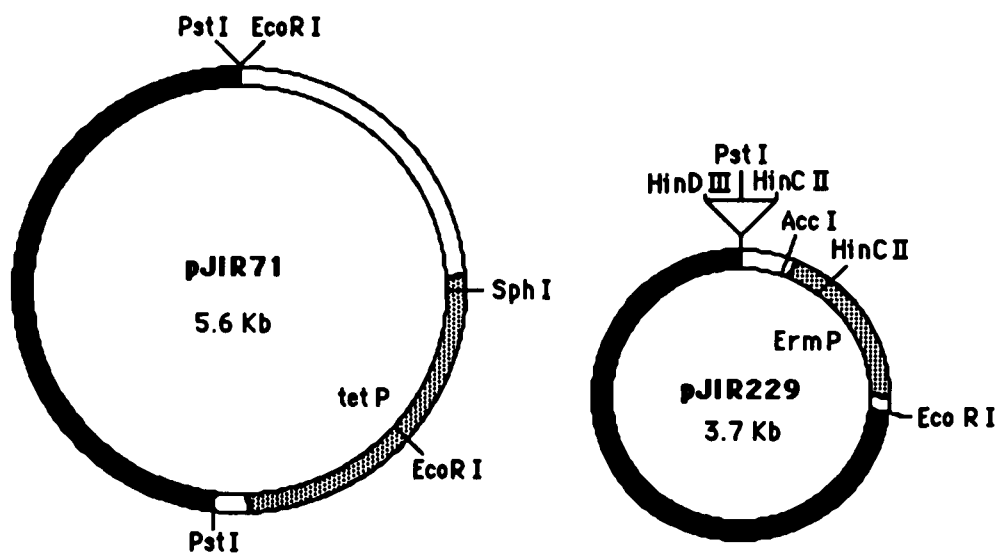


Figure 10. Restriction maps of pJIR71 (Abraham et al., 1988) and pJIR229 (Berryman and Rood, 1989). The blackened areas correspond to pBR322 (or pUC) plasmid sequences. Stippled areas designate resistance genes.

fragment on which resides a MLS^r (*ermP*) resistance gene also from *C. perfringens* (Berryman and Rood, 1989). Neither of these plasmids contain an origin of vegetative replication from *Clostridium* and therefore cannot replicate in this host. When either of these plasmids were electroporated into a Cmr *C. beijerinckii* strain already containing integral pHR106 sequences no colonies were recovered on GC plates containing either Tc or Em alone or in addition to Cm.

Cloning of antibiotic resistance genes into pHR106 and electroporation of these constructs into *C. beijerinckii*

As it became apparent that pHR106 sequences could not 'direct' the chromosomal integration of other homologous plasmids that can not normally replicate in clostridial strains, a route was sought by which the transfer and integration of pHR106 could be exploited to transfer other genetic information to *C. beijerinckii*. pHR106 was tested for the ability to transfer exogenous genes to *C. beijerinckii* after they had been cloned in the plasmid. To this end, the *tetP* and *ermP* resistance genes of pJIR71 and pJIR229, respectively, were subcloned into pHR106. Figure 11 diagrams how the 2.9 Kb fragment of pJIR71 was removed by digestion with Pst I and ligated *in vitro*

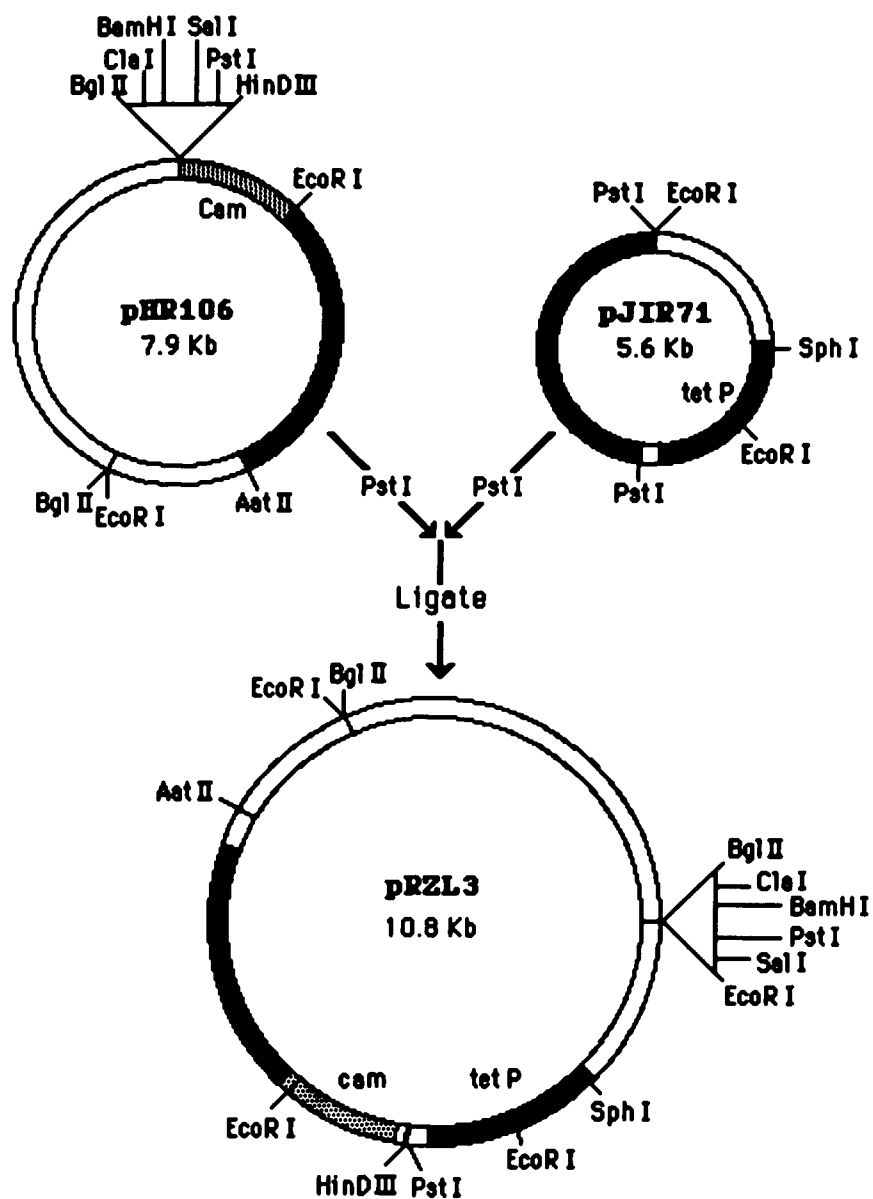


Figure 11. Construction of pRZL3 by digestion of pJIR71 and pHR106 with Pst I. The resulting 2.9 kb fragment containing the TetP gene was ligated into the unique Pst I site in the polylinker region of pHR106.

to the unique Pst I site on the polylinker region of pHR106. The resulting plasmids, designated pRZL1-5, were isolated from numerous *E. coli* HB101 transformant colonies growing on both Tc and Cm (15 and 25 µg/ml, respectively). Figure 12A shows the plasmid profiles of 5 *E. coli* HB101 isolates selected after transformation with the ligation mixture (lanes 2,3 and 5-7). The apparent molecular weight of the recombined CCC plasmids was calculated to be approximately 10.8 kb, corresponding to the expected combined MW of vector (7.9 kb) plus insert (2.9 kb). Lane 8 is a set of covalently closed circular (CCC) plasmid molecular weight markers.

The *ermP* gene from pJIR229 was recombined with pHR106 *in vivo* using a recombination proficient (*recA+*) strain of *E. coli* BD 720. Both plasmids (pHR106 and pJIR229, diagrammed in Figures 9 and 10) were sequentially introduced to this strain by electroporation. Transformants were selected for growth on Em, Cm and Amp, (the latter gene is functional in *E. coli*, but not *Clostridium*). Transformant strains harboring a single plasmid larger than the two individual plasmids were selected for further analysis. Figure 12B shows the plasmid profiles of three

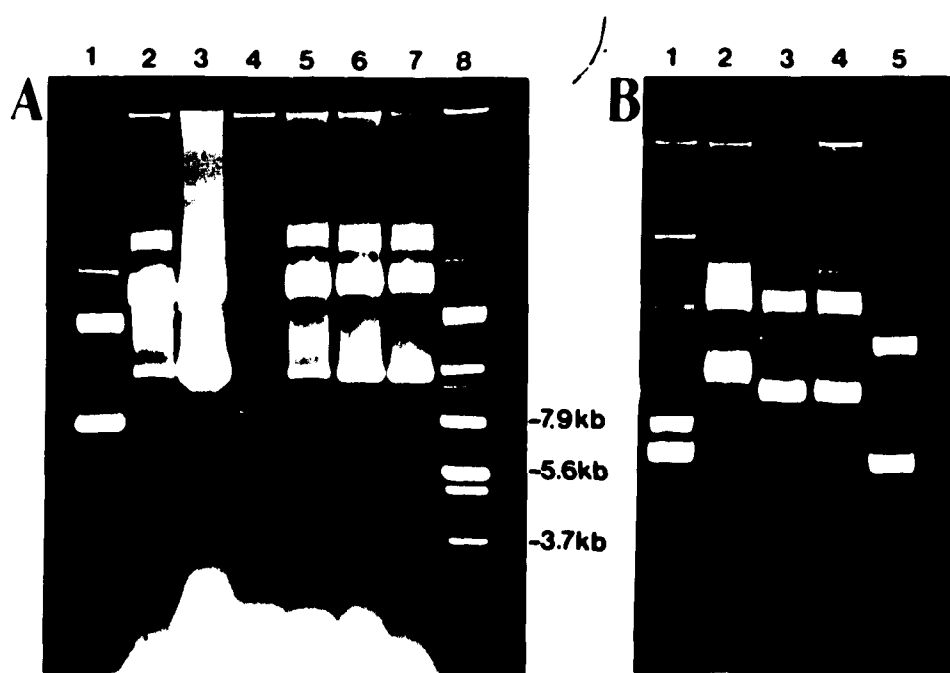


Figure 12. Agarose gel of recombinant pHR106 derivatives pRZL (A) and pRZE (B) plasmids. In gel A, lanes 2,3 and 5-7 show the plasmid profiles from 5 Cm^r transformant isolates. The lowest band in each lane corresponds to 10.8 kb, pRZL3 is shown in lane 3. Lane 1 contains pHR106 DNA, lanes 4 and 8 contain CCC molecular weight estimators. The sizes indicated to the side are for; pHR106 (7.9), pJIR71 (5.6), and pJIR229 (3.7). Photograph B lanes 2-4 are three Em^r transformant isolates, pRZE2, 4 and 6. The vector pHR106 is run in lane 5.

transformants harboring such large plasmids designated pRZE2, 4 and 6 (lanes 2-4). Plasmids pRZE4 and 6 seem to have undergone deletions resulting in the loss of an approximately 1.6 kb fragment. These plasmids therefore are smaller than pRZE2, which is approximately 12.2 kb, the size corresponding to the additive sizes of pHR106 and pJIR229. Due to its slightly smaller size pRZE4 was chosen for further use in characterizing the ability of pHR106 sequences to express or integrate cloned sequences in *C. beijerinckii*.

The plasmid constructs were then individually electroporated into *C. beijerinckii* NRRL B-592. In these experiments, the parent plasmid pHR106 was also electroporated as a positive control. Viable counts were done to enumerate each population before and after the electric pulse was applied. Both pRZE4 and pRZL3 were observed to transfer to the wild-type strain at approximately the same frequency as pHR106 (1×10^{-7}). Colonies resistant to Em and Cm arose on GC plates after pRZE4 was present in cuvettes subjected to the same pulse conditions as was used to transfer pHR106. No Tcr colonies were selected after electro-transformation with pRZL3, although Cmr colonies were selected. Cmr isolates

suspected of harboring pRZL3 sequences were subcultured in Tc-containing broth (0.5 µg/ml) overnight and then transferred to GC plates with selective levels of Tc. These cultures then displayed full Tc resistance (10-20 µg/ml) and would grow in the presence of both Tc and Cm. Experiments were also performed in which *C. beijerinckii* Cmr strain C14 (containing integral pHR106 sequences) was electroporated in the presence of either pRZE4 or pRZL3. In either case after three tries, no Tc or Em colonies were observed even though the number of cells surviving the pulse was significantly greater than 1×10^7 CFU/ml, indicating sufficient cell numbers to detect transfer.

Localization of plasmid constructs pRZL3 and pRZE4 after transfer to *C. beijerinckii*

The behavior of pHR106 after transfer to wild-type *C. beijerinckii* NRRL B-592 and the similar transformation frequencies exhibited by pRZE4 and pRZL3 indicated that they may also be integrating into the chromosome. Therefore, chromosomal DNA was extracted from Em^r and Tc^r transformant strains, digested with Xba I and separated by agarose gel electrophoresis (Figure 13A and C). Lanes 4 and 6 in Fig. 13A and C are the Tc^r (pRZL3) and Em^r (pRZE4)

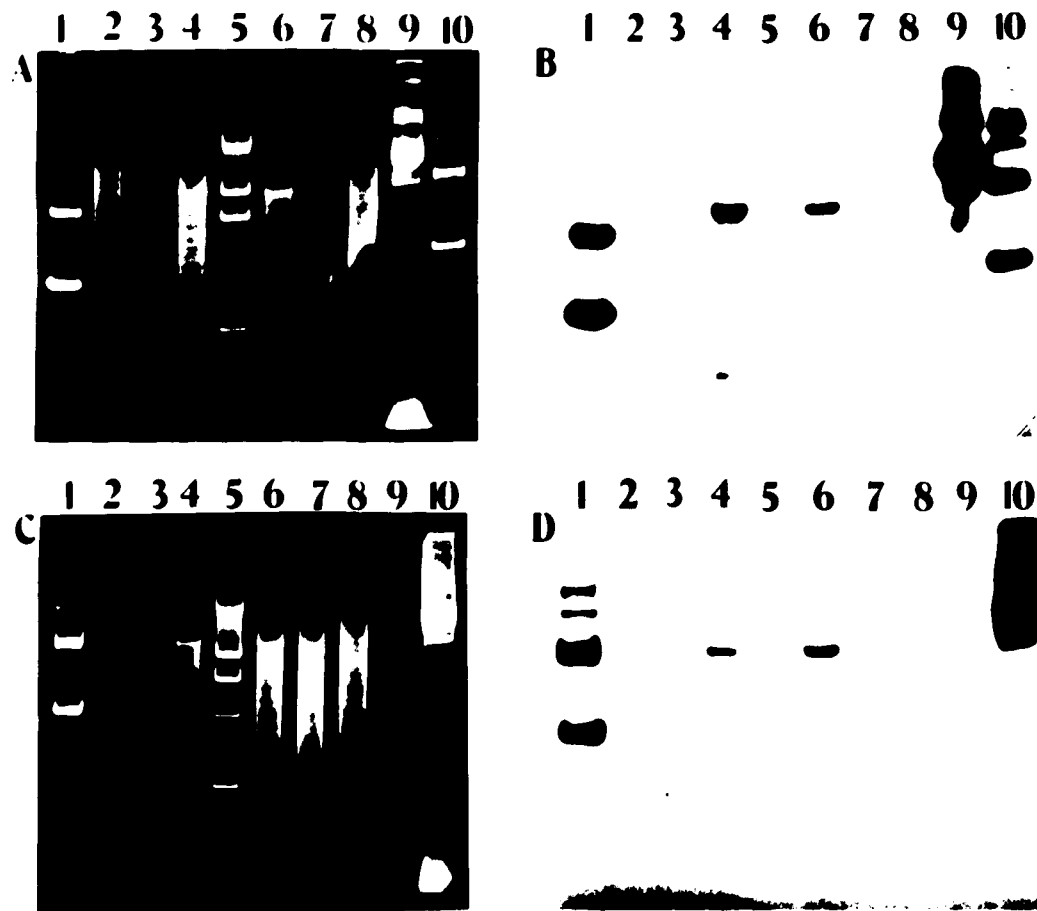


Figure 13. Agarose gels (A and C) and corresponding Southern transfers probed with either pRZL3 (B) or pRZE4 (D). In A and C, lanes 2,3 and 7,8 contain *Cm^r* transformant C14 and *C. beijerinckii* wild-type *Xba* I digested DNA, respectively. Gel A, lanes 4 and 6 contain similarly treated DNA from a *Tc^r* transformant. Lanes 4 and 6 in gel C contain the same from an *Em^r* transformant of *C. beijerinckii*. pHR106 plasmid DNA was run in lane 10 (gel A) and lane 1 (gel C) and Lambda *Hind* III restriction fragments were run in lane 5 in both gels. Lane 9 in gel A contains pRZL3 plasmid DNA, and lane 10 gel C contains pRZE4 DNA. Lastly, lane 1 of gel A contains pJIR71 plasmid DNA.

transformant DNA samples, respectively. In each case particular bands stand out from the regular chromosomal bands, cf. wild-type (lanes 3 and 7 in both figs.) or Cmr C14 strain (lanes 2 and 8 in both figs.) Note that there are no such darkly staining bands in the lanes containing Xba I-digested chromosomal DNA of strain C14 (lanes 2 and 8 on either gel) known to contain integrated pHR106 sequences. The migration distances of these bands in each case correspond roughly to the migration distance of the CCC plasmid bands of pRZL3 and pRZE4 included on these gels (Fig. 13A, lowest band, lane 9 [CCC pRZL3]; Fig. 13C, third band from top, lane 10 [CCC pRZE4]). Figure 13B and D show the autoradiograms of Southern blots of these gels probed with ³²P labelled pRZL3 and pRZE4 plasmid DNA, respectively. In each case the unique darkly staining bands showed homology to their respective probes. Also, as expected, the Cmr strain C14 lane containing integral pHR106 sequences showed homology to the probes, and the wild-type did not.

Since the above data shown in Fig. 13A-D indicated the recombined pHR106 derivatives were no longer integrating into the chromosome of *C. beijerinckii*, an experiment was carried out to test this. Since linear DNA has no transforming activity samples of the

chromosomal DNA preps described above were individually electroporated to *E. coli* HB101. *E. coli* transformants were selected that were resistant to Cm, Em, and Tc, when DNA from strain C14, the Em^r *C. beijerinckii* transformant, and the Tc^r *C. beijerinckii* transformant, respectively, were used. Subsequent plasmid extractions from these *E. coli* transformants revealed the presence of the three respective plasmids. Each of the plasmids were shown to be the correct size with respect to each other.

IV. DISCUSSION

Growth rate, metabolism and competence of *B. subtilis* in continuous culture

It has been repeatedly reported in the literature that competence occurs in batch cultures of *B. subtilis* in late-log, early-stationary phase, when most of the culture's growth has ceased (Dubnau, 1991). While the former statement is undeniably true for batch culture studies, the latter dogmatic assertion loses meaning when competence is studied in continuous culture. For example, we consistently observed steady state cultures of *B. subtilis* to maximally express competence at the rapid growth rate of 2.5 h (t_d). Competence for transformation with plasmid, bacteriophage or chromosomal DNA showed the same pattern whether the continuous culture was carbon or nitrogen limited. Some of these findings corroborate those of Portoles et al. (1977), who, in addition to describing the competence peak at 2.5 h, also reported a second lesser peak of competence in chemostat-grown cells at a t_d of about 7.0 h. Throughout the work presented here, a second competence

event was not detected, even when *B. subtilis* was cultured at ultra-slow growth rates in a biomass recycling fermentor. Portoles et al. (1977) did, however, report the secondary competence peak disappeared when competence was either measured by transfection, or measured by transformation to prototrophy after L-glutamic acid was added to Bott and Wilson's (1967) transformation medium (Portoles et al., 1977). They also reported the two peaks responded differently to various amino acids added to the transformation medium and implied that this was evidence of two separate routes of competence development in *B. subtilis* (Lopez et al., 1970). No additional reports have appeared that describe alternate routes to the competent state.

The growth rate ($2.5 \text{ h } t_d$) of maximal competence would roughly correspond to batch culture growth rates that, while rapid, would be sub-maximal and would not be realized in a glucose-casamino acids competence medium until a nutrient was exhausted. This would cause the growth rate to deviate from log (maximal) growth and then fall past a rate of 2.5 h where competence is expressed. The fact that competent cells can be recovered from steady state chemostat cultures demonstrated that these cells

necessarily had to be growing prior to their differentiation to the competent or other 'pre-competent' state. In a sense, this may eliminate the potential argument that there is more than one population (eg. pre-competent and non-competent) in the growth vessel at any given time (or steady-state growth rate). Since it is known that a *B. subtilis* cell expressing competence is relatively metabolically inactive compared to a non-competent cell (Dooley et al., 1971), competent cells by definition must wash out of the growth vessel. To detect a steady state level of competent cells the rate of (pre) competence development must exceed the washout rate. Therefore, from a growth rate perspective, the culture can be considered homogeneous until the cells express competence. Metabolically, however, the question of homo or heterogeneity in steady state chemostat growth is more complicated. During growth in batch culture the population rapidly falls past the growth rate of maximal competence expression producing two metabolically distinct populations. Batch cultures thus are inherently poor growth systems for studying complex physiological processes. When *B. subtilis* cells grow in a steady state at a t_d of 2.5 h, every cell must either grow at this rate or wash out. At any given time any cell

could theoretically depart from 'non-competent' growth and become competent. Therefore, any observed change in the steady state culture should be extrapolatable to cells that are differentiating as well as to the 'non-competent' cells.

Carbon/energy-limited *B. subtilis* cells grown in continuous biomass recycling fermentor culture showed a changing pattern of metabolite secretion. At growth rates faster than 2.5 h (t_d) the culture excreted acetic acid in a 1:1 stoichiometric ratio to the limiting glucose (4 mM). When the mass doubling time of the culture exceeded 2.5 h, the concentration of acetate in the medium reduced tenfold and remained at a concentration of about 0.4 mM .

It has been established that *B. subtilis* does not possess a fully oxidative tricarboxylic acid (TCA) cycle during exponential growth in a glucose-based minimal or complex medium (Hanson et al., 1973; Szulmajster and Hanson, 1965). TCA enzymes such as aconitase and citrate synthase, (those of the tricarboxylic arm of the cycle) are repressed by glucose (Sonenshein, 1989). Batch culture sporulation studies of *B. subtilis* have shown the medium initially becomes acidified during exponential growth and then returns to neutral shortly after the transition to stationary phase (Szulmajster and

Hanson, 1965). This is due to the excretion of organic acid products of glucose catabolism such as acetate and pyruvate during log growth which are later used at the end of exponential growth (when the TCA cycle functions in a fully oxidative capacity) (Hanson et al., 1973).

It is now known that while the induction of an oxidative TCA cycle is concomitant with sporulation and competence development in batch cultures, the various enzymes for the TCA cycle and the two differentiation processes have been placed in separate regulatory groups based on their response to the presence of glucose in various media (Sonenshein, 1989).

B. subtilis also possesses a little known and studied butanediol cycle (Lopez and Fortnagel, 1972). The pathway allows growth on acetoin (acetyl methylcarbinol) or 2,3- butanediol as sole carbon source, with acetate being the primary end product, and NAD⁺ being reduced fermentatively (Lopez and Fortnagel, 1972). This pathway has been shown to be operative whether the cells are grown aerobically or anaerobically (Juni and Heym, 1956). However, there is scant information as to the functioning of this cycle when cells are grown on glucose.

Recently, Bulthuis et al. (1989) have demonstrated that steady-state chemostat cultures of *Bacillus licheniformis* will exhibit spill, or overflow, metabolism when growing at near maximum rates. They observed a decrease in the molar growth yield (Y_m) at all growth rates faster than a t_d of 2.3 h ($\mu = 0.3$). Also present in the medium during this fast growth rate range were spill products such as malate, keto-glutarate, and citrate, all present due to the under-oxidation of glucose. Some types of overflow metabolism have been characterized by Neijssel and Tempest (1975). It is an adaptation to a severely high demand for ATP needed to support equally high rates of anabolic synthesis. This process is an alternative to other manifestations of spill metabolism such as the deletion of phosphorylation sites or a reduction in the P/O ratio (Stouthamer and van Verseveld, 1985). Apparently, due to a rapid rate of growth, the cells simply require energy for biosynthetic processes at a higher rate than can be supplied by normal metabolism where complete glucose oxidation is realized. The solution is a partial uncoupling of energy generating oxidative pathways from anabolism. This allows a faster (yet less energy efficient) supply rate of ATP to accomodate the cell's rate of biosynthesis (Stouthamer and van

Verseveld, 1985). In this manner, cells growing at rates anywhere between maximal and a t_d of 2.5 h can be considered anabolically limited, and those cells growing at slower rates are catabolically limited. In any case, a cell traversing this critical growth rate range must significantly shift the flow of carbon and energy between the differing purposes of assimilation, dissimilation, and other areas such as exocellular product formation (and competence development?) (Bulthuis et al., 1989; Frankena et al., 1985). It is interesting that the switch from anabolic to catabolic limitation, or from spill to fully oxidative metabolism, in *B. licheniformis* occurred at the same t_d as was observed to occur in *B. subtilis*. I determined that the main spill product of *B. subtilis* was acetic acid. Acetate was produced and excreted at growth rates in excess of 2.5 h. At the slower rates acetate was either used for energy production as has been previously reported in batch culture studies (Speck and Freese, 1973; Szulmajster and Hanson, 1965; Lopez and Fortnagel, 1972), or was no longer produced. Spill metabolism at rapid growth rates is not unique to *Bacillus*, Andersen and von Meyenburg (1980) have demonstrated that aerobic batch cultures of *E. coli* also secrete acetate which is later completely oxidized after

the glucose is exhausted from the broth.

Data from nitrogen-limited chemostat cultures of *B. subtilis* established at various steady-state growth rates (Fig. 2) provide additional evidence of spill metabolism occurring at fast growth rates and ending at a t_d of 2.5 h. Cultures established at t_d lesser or greater than 2.5 h reached lower steady state biomass concentrations (X mg/ml). The highest X value occurred at precisely the growth rate when competence is seen to develop, at the apparent metabolic switch point from anabolic to catabolic limitation. Barring O_2 limitation, the low X values observed at the rapid t_d (< 2.5 h) can be explained by a low efficiency of energy production needed for anabolism that would occur during spill metabolism. Oxygen limitation may not be responsible for this observation for two reasons. First, oxygen sparging and medium agitation rates were tested to be in excess of what was needed to support the cell density in the growth vessel of the chemostat (during steady state operation a doubling of the agitation rate did not result in an increase in cell density); second, at these fast t_d *B. subtilis* cells have been shown to consume less O_2 per cell than when the TCA cycle is fully operative (Szuljmaster and Hanson, 1965; Srinivasan,

1965; Halvorson, 1962).

Spore levels (phase bright, phase dark, and free spores) were observed to rise above 1% at about the time of competence development (Fig. 4). Spores were also detected by microscopy counts at the faster growth rates, only their numbers were below 1%. Sporulation under all culture conditions was unlike the acquisition of competence in that there was no bulk sporulation event. The rate of appearance of spores was inversely related to the growth rate of the culture whereas the appearance of competence was a single event. Dawes and Mandelstam (1970) have previously shown similar sporulation kinetics in chemostat cultures of *B. subtilis*.

It appears, then, that during the relatively unrestricted and rapid growth rate of about $2.5 \text{ h } t_d$, *B. subtilis* undergoes a profound metabolic adjustment. This reshuffling of carbon flow, which can be thought of as a switch from anabolic (energy) to catabolic (carbon) limitation occurs at precisely the growth rate at which a subpopulation of the culture begins to develop competence. The strict dependence of the development of competence on a particular growth rate (or stage in batch cultures) has never been 'unlinked' by

mutation (Dubnau, 1991). Furthermore, given an acknowledged lack of any significant physiological event which could be implicated as the *first* signal to the cell that a state of nutrient starvation may be imminent (Dubnau, 1991), I propose the idea that the switch from spill to a fully oxidative metabolism as growth rate slows may be such a cellular indicator.

Formation and regeneration of *C. beijerinckii* NRRL B-592,

B-593 and *C. acetobutylicum* ATCC 10132 protoplasts

The original purpose of this work was to develop a protocol and medium that would yield stable protoplasts of *C. beijerinckii* B-592 and allow the protoplasts to regenerate cell walls. In addition to meeting these objectives, a second medium was formulated that permitted protoplasts to replicate into colonies of cell wall-deficient, osmotically sensitive forms that could be transferred to the regeneration medium on which they gave rise to the bacillary form.

The method for forming protoplasts from *C. beijerinckii* cells described here is similar to the method reported for *C. acetobutylicum* by Reilly and Rogers (1987). Their protocol used 50 mM $\text{CaCl}_2 + \text{MgCl}_2$ in the protoplast formation broth because this

concentration allowed the highest frequency of regeneration. For *C. beijerinckii*, 12.5 mM or greater concentrations of these salts resulted in stable protoplast formation, but higher concentrations did not increase the frequency of L-colony growth or regeneration. Optimum protoplast formation occurred when rapidly growing cultures were treated with lysozyme in CBM medium (with 0.4 % glycine) containing 0.5 M sucrose and 12.5 mM $\text{MgCl}_2 + \text{CaCl}_2$.

It was possible to cultivate lysozyme-generated protoplasts of *C. beijerinckii* on BLM as wall-deficient, or L-, colonies. These colonies, when removed as agar plugs and spread onto fresh BLM plates, gave rise to numerous other L-colonies which continued to grow as L-forms through at least 3 passages without reverting to the bacillary state. However, L-colonies transferred from BLM to BRM plates either regenerated cell walls 25 % of the time or continued to slowly grow as L-colonies. The only report of clostridial L-forms and colonies was by Heefner et al. (1984). They transformed autoplasts and L-phase variants of *C. perfringens* with plasmid DNA, but only the autoplasts would regenerate to rod-shaped cells.

The only difference between BLM and BRM is the concentration

of the CaCl_2 and MgCl_2 . It seems that high concentrations of these salts (37.5 mM in BLM) support the growth of these strains as wall-less colonies. The need for high concentrations of these salts in clostridial protoplast regeneration media has been shown before (Allcock et al., 1982; Reilly and Rogers, 1987; Reysset et al., 1987; Stahl and Blaschek, 1985). Reilly and Rogers (1987) observed the highest frequencies of regeneration of *C. acetobutylicum* when protoplasts were formed in the presence of 50 mM CaCl_2 and MgCl_2 and then plated on a soft agar medium containing 25 mM concentrations of these salts. A similar situation with respect to L-colony regeneration was observed for *C. beijerinckii*. L-colonies were induced to regenerate when plated on the threefold lower concentrations of CaCl_2 and MgCl_2 in BRM.

Protoplasts of *C. beijerinckii* NRRL B-593 and *C. acetobutylicum* ATCC 10132 showed a growth and regeneration pattern on BLM and BRM similar to that of B-592 for which these media were formulated. These strains grew as wall-deficient colonies on BLM and reverted to the bacillary form when transferred to BRM. *C. beijerinckii* B-593 formed fewer L-colonies per input protoplast but regenerated at a frequency comparable to that of

B-592. Interestingly, *C. acetobutylicum* ATCC 10132 formed L-colonies at frequencies equal to B-592 and regenerated at only a slightly lower frequency (Table 3). Although *C. acetobutylicum* strain 4259 formed >99 % protoplasts by this method, it failed to grow on either BLM or BRM, even when untreated cultures were plated directly.

Protoplast regeneration among clostridia has been cited as being species-specific (Reysset et al., 1987). The procedure described here is the first that allows regeneration of protoplasts from more than one species of *Clostridium*; however, strains of the same species varied in their ability to regenerate to the bacillary form when this protocol was used.

Protoplast transformation and fusion studies with *C. beijerinckii* and *Clostridium* C7

Protoplast transformation experiments in which various plasmids of Gram positive origin were tested did not result in successful transfer and/or expression of any of these plasmids in *C. beijerinckii* NRRL B-592. This result may not represent a failure of the above mentioned protoplast regeneration procedures to be adaptable to PEG-mediated plasmid transformation. Rather, it may

simply reflect the fact that none of the plasmids tested could be transferred to *C. beijerinckii* at a frequency greater than 1×10^{-7} , if at all. Based upon protoplast survival and regeneration frequencies, this frequency would be well below that needed to detect transfer and/or expression of any of the aforementioned plasmids, including pHR106, which was shown to be expressed in *C. beijerinckii*.

Protoplast fusion experiments using the same protoplast formation and regeneration procedures were also terminated due to unsatisfactory results. While the fusants phenotypically exhibited some of the desirable traits and at first appeared to be recombinants between *C. beijerinckii* and *Clostridium* C7, it was not possible to unequivocally demonstrate this on the genetic level. The extremely high GC ratios exhibited by the fusants, while possible in theory (GC-rich areas of the respective parental genomes could have recombined), are extremely unlikely. Likewise, the data from DNA-DNA liquid hybridization studies of fusant F4 DNA (Table 10) does not offer strong evidence that it is a bonafide fusant. The average percent homologies of its DNA to that from both C7 and *C. beijerinckii* should add up to approximately 100% (if it indeed contains sequences derived only from these sources). However,

these numbers add up to about 44%. One explanation for these results is that due to contamination a third, unrelated, organism was involved in the fusion process. The virtual absence of selective pressure in the protoplast formation broth, L-colony and regeneration plates lends support to this conclusion (neither solid medium would operate as developed when certain antibiotics were added). However, there is evidence that recombined strains were produced and therefore fusion was accomplished. Results showed the type III fusant retained all the major phenotypic characteristics of *C. beijerinckii*, including production of solvents, while having a widely divergent G-C ratio from that of *C. beijerinckii*, indicates that it likely arose via fusion with an unknown organism.

The protoplast formation and regeneration procedures devised for use with *C. beijerinckii* and a strain of *C. acetobutylicum*, may still be adaptable to PEG-mediated genetic recombination protocols. What is needed to test this is a plasmid that transfers to and is expressed in *C. beijerinckii* at a higher frequency. Similarly, in order for protoplast fusion studies to be feasible using *C. beijerinckii* and the above system perhaps a more closely related bacterium should be chosen. Certainly, multiply antibiotic resistant

strains of both parents are needed, provided the antibiotics do not inhibit the ability of any of the protoplast, or L-colony growth media to osmotically support the protoplasts.

Electro-transformation of *C. beijerinckii* NRRL B-592 with shuttle plasmid pHR106 and recombinant derivatives

C. beijerinckii NRRL B-592 was transformed after plasmid pHR106 DNA was electroporated into intact cells. Several attempts to recover the plasmid from the cytoplasm were unsuccessful and Southern hybridization procedures confirmed that the plasmid had integrated into the chromosome. The integration occurred at a frequency of 1×10^{-7} (per viable cell after the electric pulse) and apparently in a non-random fashion. The latter observation precludes the possible use of pHR106 as a random insertional mutagen in this organism. This also indicates that there is a specific integration site, perhaps similar to an IS element directing the insertion of the vector. Sequences present on pHR106 but not expressed in *C. beijerinckii* (pBR322) were also found to be present in the chromosome after transfer. The integrated pHR106 sequences would not permit the integration of other plasmids sharing sequence homology with it, nor would it allow the transfer or expression of

insert-containing derivatives pRZE4 or pRZL3. When either of these derivatives were independently transferred to the wild-type *C. beijerinckii* they appeared to no longer integrate into the chromosome. Results from agarose gel electrophoresis of Xba I digested chromosomal DNA from the transformants showed darkly staining bands not apparent in lanes containing similarly treated chromosomal DNA from the Cmr transformant strain C14. The darkly staining Xba I fragment seen in lanes containing Emr (containing pRZE4) transformant DNA, when measured using λ HindIII restriction fragments as MW standards, measured 11.2 kb. pRZE4 has been calculated to be about 10.0 kb in size (measured in CCC supercoiled form with appropriate MW markers). This plasmid could be expected not to integrate after being generated by *in vivo* recombination in a *rec+* *E. coli* host. It is possible but has not been proven that the same site on pHR106 that causes integration into the *C. beijerinckii* chromosome could have caused pHR106 to recombine with pJIR229.

The two darkly staining bands in lanes containing Xba I digested Tcr transformant DNA showing homology to pRZL3, when similarly measured, correspond to 9.5 and 2.1 kb. This gives an additive MW of about 11.6 kb. The MW of pRZL3 has previously been

calculated to be 10.8 kb. In this case, since there are two fragments that hybridized to pRZL3, a restriction analysis of this plasmid may help to show whether or not the plasmid has indeed integrated (the plasmid will contain only 1 Xba I site) or is present in the cytoplasm of *C. beijerinckii* as an autonomously replicating vector with 2 Xba I sites. Chromosomal DNA samples from each type of *C. beijerinckii* transformant, ie. containing pHR106, pRZE4 and pRZL3 sequences, were successfully transformed into *E. coli* HB101. Because HB101 is deleted for most recombinatorial functions, and linear DNA transforms cells poorly at best, these results are interpreted as showing that all three plasmids can and do reside in the cytoplasm in the autonomous state. In the case of pHR106 it appears that it can either integrate into the chromosome of *C. beijerinckii* or can excise and replicate autonomously. The recombinant pHR106 derivative plasmids have not been observed to integrate into the chromosome under any circumstances.

In the construction of pHR106, Roberts et al. (1988) used clostridial DNA from two different sources. The clostridial origin of vegetative replication (oriV) was provided by a cryptic plasmid from *C. perfringens* 12502. The chloramphenicol resistance gene

was subcloned from pJIR62. pJIR62 is essentially pUC18 containing a 1.5 kb region of *C. perfringens* transposon Tn4451 containing the entire Cmr gene, but not the transposon termini (Abraham et al., 1985). Since the Cmr gene was originally carried by Tn4451 the possibility exists that sequences within or flanking this gene in pHR106 may be promoting integration in *C. beijerinckii* NRRL B-592. Interestingly, Abraham and Rood (1987) could not rule out the possibility that sequences within the Cmr gene proper were affecting transposition in *C. perfringens* and *E. coli* hosts.

Regardless of whether the insert-containing pHR106 derivatives integrate into the chromosome, it is significant that this plasmid was shown to be a useful cloning vector for *C. beijerinckii*. It was demonstrated that genes could be cloned into pHR106 and subsequently transferred to *C. beijerinckii* where they were expressed. Although the transfer frequency to *C. beijerinckii* is low, pHR106 is a shuttle vector and will also operate in *E. coli*. Therefore, any genes to be cloned into *C. beijerinckii* can first be cloned into pHR106 in an *E. coli* host, where the transformation frequency is equal to that of the pUC or pBR series of vectors. Once in *E. coli*, the particular clone can be screened, selected and purified.

Then the purified recombinant plasmid may be transferred to *C. beijerinckii* where it will only be necessary to select a single colony. This is the first description of a feasible plasmid-based cloning system for *C. beijerinckii*. Further molecular analysis is necessary to clarify the events leading to the integration of pHR106. Work of this nature was outside the scope of the present study, but is certainly meritorious of further inspection.

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